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MUTATIONS

Second Conference on Genetics

Edited by
WILLIAM J. SCHULL

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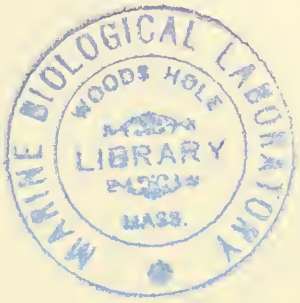
PREFACE

Somewhat over twenty years ago, the Josiah Macy, Jr. Foundation initiated a series of regularly scheduled conferences directed toward challenging problems in medicine and health. With time, firm but not immutable ground rules have been evolved for the conduct of these sessions. Thus, each conference group is to meet annually for a period of five or more years. Twenty-five persons, selected to represent a multidiscipline approach, are to participate in a meeting. These individuals are termed conference members, if they have been selected to participate in all of the meetings of a group, and guests, if they are to participate in a single meeting.

The purpose of each conference is the promotion of communication, the exchange of ideas. To this end, an informal give-and-take among the participants, members and guests, is encouraged. Structure and continuity are given the discussion by a leader whose function is to present some of the more interesting aspects of the problem under discussion. The participants are enjoined to interrupt this presentation with questions, criticisms, and comment. At their best, the interruptions lay bare the birth and maturation of an idea, and form, therefore, an essential part of the lessons to be gained from the conference process. To share these lessons as widely as possible, an edited transcript of the meeting is published. These transactions, which attempt to retain the spontaneity of the discussion, have aroused considerable interest and criticism. Comments range from an enthusiasm for, to a total rejection of, the personalized approach. Criticism, in the words of Frank Fremont-Smith, for many years the guardian of these conferences, "has been directed primarily to editorial permissiveness which has allowed in the final text, in some instances, too many questions, remarks, or comments which, although perhaps useful during a heated discussion, seem out of context and interrupt the sequence of thought." Clearly, not all critics recognize the narrowness of the path twixt spontaneity, on the one hand, and editorial permissiveness, on the other, nor the challenges which confront the editor.

CONTENTS



PARTICIPANTS ix

PROBLEMS OF MEASUREMENT OF MUTATION RATES 1

Presented by K. C. Atwood

Group Interchange

Summary by Theodosius Dobzhansky 72

References 74

MUTAGENESIS, WITH PARTICULAR REFERENCE TO CHEMICAL FACTORS 78

Presented by Charlotte Auerbach

Group Interchange

Mutagenic Effects of Manganese Chloride—M. Demerec 115

Genetic Fine Structure of Phage T4—S. Benzer 136

Summary by M. Demerec 157

References 160

MUTAGENS CURRENTLY OF POTENTIAL SIGNIFICANCE TO MAN AND OTHER SPECIES 167

Presented by Avram Goldstein

Group Interchange

Summary by S. Zamenhof 233

References 238

Index 243

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October 16-19, 1960, Princeton, New Jersey

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PROBLEMS OF MEASUREMENT OF MUTATION RATES

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Mutations, as everybody knows, are changes in nucleotide sequence. The spontaneous rates of such changes have been estimated in different organisms with varying degrees of indirectness. I would like to compare the situation with respect to such estimates in man and in simpler organisms.

This graph (Fig. 1) shows two distributions of reported mutation rates. The group over here (open bars) is in man, and the group here (solid bars) in microorganisms. The most frequent reported rate in man is between 10^{-4} and 10^{-5}.

Freese: Per what?

Atwood: What it is per is really not clear, as you will see, but it is commonly expressed per gamete, or per human generation. In microorganisms the usual rate is somewhere between 10^{-9} and 10^{-8} either per generation or convenient time unit, which is usually some time not very different from generation time or doubling time.

Auerbach: Per locus?

Atwood: Whether it is per locus is not known certainly for all cases. I have included mistakes that may have been made, as well as any valid rates that happen to be in the group.

Demerec: How did you get these data? Are they all from the literature?

Atwood: Yes. There are about 50 estimates in microorganisms, about half of which were reported by the Carnegie group.

Glass: Are these per cistron, or per microbial group, or per site or what?

Atwood: They are mixed. I have not excluded any categories except those where a mutator gene was known to be involved or in which there were clearly labile alternative states, such as phase variation and things of that sort, that tend to have high rates.

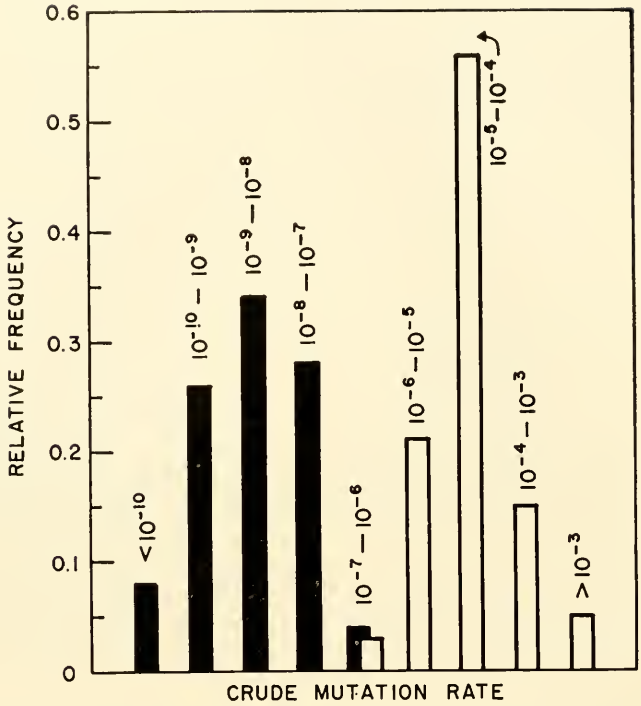

Figure 1. Distribution of 50 bacterial and 39 human crude spontaneous mutation rates. Solid bars = bacterial; open bars = human. The rates are not expressed in comparable units; human rates are per human generation and bacterial rates per bacterial generation, or approximate equivalent.

Auerbach: Did you include only those which were obtained by a screening technique or are so-called forward mutations also included?

Atwood: In microorganisms most of these are back mutations. Those that are not back mutations are mostly phage-resistance.

Demerec: It makes a great deal of difference what kind of mutations you are considering. Back mutations are changes, presumably, in one specific site, whereas forward mutations may be changes in any one of a great many sites.

Atwood: Yes. The apparent rates in man are higher than in microbes by about a factor of 10^4, and a number of things may contribute to this factor. One is the one you just mentioned, that if you have a

certain number of sites, a change in any one of which gives the phenotypic change being screened, then the forward and back mutation rates should differ by a factor representing the number of sites.

Of course, the fact that not all the sites have the same rate will make this only an approximation, but it is probably a very good one.

Benzer: Are you trying to give an explanation of why human rates are higher?

Atwood: Yes. One reason is that, usually, we can surmise that they represent loss mutations or forward mutations; in other words, one would be, in your system, looking at the rate only from r^+ to r, rather than any of the reverse rates. What is the difference between the overall mutation rate r^+ to r and the average of the various r's back to r^+, for example?

Benzer: There is no average reversal rate.

Atwood: The reversion rates vary all the way from nondetectible to about several per cent per growth cycle?

Benzer: It is a range of 10^6.

Atwood: How many possible r's are there?

Benzer: At least 500.

Atwood: All right; that's the factor I'm looking for.

Auerbach: Would it be possible to compare forward and reverse mutation rates? The latter is much lower in Drosophila.

Atwood: Well, I was able to find only one example of forward and back mutation rate measured with respect to nutritional requirement in bacteria, and that is the h$-\!\!\rightleftarrows$h+ histidine system in which Lieb(20) was able to get an estimate of the rates in both directions. The difference was about a hundredfold.

Goodgal: You're only considering one locus in one gene. Seymour points out that the reversion rates vary by a factor of 10^6.

Atwood: Does anyone wish to maintain that these factors are meaningless in the sense that there shouldn't be any difference?

Lederberg: I'll accept your challenge. You get a very wide dispersion of rates in both cases, but they overlap so much that it's hard to know what particular conclusion you should be able to draw.

Zamenhof: The unstable genes are not as infrequent as one might think, and there is no evidence that the instability is caused by a mutator gene in every case. A frequency of 10^{-4} or 10^{-2} is very often found, if one uses the proper techniques (44).

Demerec: May I raise a question? How frequent are hot spots in phages? Our experience with Salmonella indicates that if they occur at all in this bacterium their frequency is considerably lower than that

observed by Benzer in the *r* II region of phage T4. For example, intercrosses involving 52 leucine mutants showed that the mutations had occurred at different sites of the locus (12). Occasional repeats have been found, but even those are not frequent. Certainly their frequency is not as high as that reported in phage. What I have in mind is the question of whether hot spot behavior is characteristic for phage, but not of organisms on a higher level of organization, and this would include bacteria. Is there any evidence for hot spots in organisms higher than phage?

Benzer: Such information is very difficult to get in higher organisms because you cannot easily distinguish between mutations at the same site or at closely adjacent sites.

Demerec: In bacteria, we should be able to detect them.

Lederberg: I would like to answer the question from our experience. I think it depends on which gene you're looking at. In the *gal* region, some 100 or 200 loci have been examined, and there are no known recurrences there. In the *lac* region, there are at least five or six recurrences at one particular locus, in a sample of 60 or 70 mutants; so that is not a very hot spot. But there is certainly a difference in the behavior of the material at these two loci.

Atwood: I wanted to mention the well known causes of mistakes in human mutation rates. An equilibrium is commonly assumed between gain of mutant alleles by mutation and loss by selection; the rate determinations usually involve three factors. The frequency of affected individuals is one; then, a relative fertility factor, and then another factor which is really the ratio of the alleles per person subject to selection to the alleles per person subject to mutation. For an autosomal recessive, this is unity; for an autosomal dominant, ½, and for a sex-linked recessive, ⅓.

Ignoring these constants we could be off by a factor of 3 at most, so they may seem a needless embellishment when you consider the difficulty of measuring relative fertility, ascertaining affected individuals without bias, and evaluating the assumption that the population is in equilibrium.

One can guess about how far off these estimates could be because of such uncertainties. Where inbreeding has decreased so that gene frequencies are not at equilibrium, the error would almost surely be less than tenfold. Take an extreme case, for example of one per cent inbreeding with an equilibrium gene frequency of 10^{-3}. The homozygotes would then be about 10^{-5}. If inbreeding suddenly stopped, the homozygotes would drop temporarily to 10^{-6}.

Another factor is the selection of unusually high rates for study.

The human markers that might have been used for mutation studies are much more numerous than those that have been used, so some kind of selection has gone on. However, extreme rarity is only one of many reasons not to estimate the mutation rate. I think that the rates reported may be fairly representative.

Auerbach: May I ask a question? Do you feel sure that, for those which have been studied, only one locus is involved? That is a big assumption.

Atwood: No, that is never certain. I don't know whether there is any point in more discussion of how badly mistaken we can be with respect to locus plurality, phenocopies, penetrance, somatic mutation, or other things that give spurious frequencies of affected individuals. These are known to everyone, and they are, I think, really impossible to assess.

As to the determination of relative fertility, there have been different opinions about what controls are appropriate. An apparent mutation rate has been revised by as much as a factor of 5, depending on which extreme view was adopted (35).

So we have an unknown amount of uncertainty caused by things other than gene frequency that influence the frequency of affected individuals, and possibly a factor of 5 due to uncertainty of the relative fertility, and factors of from 100 to 1000 involved in the direction of the mutation which in man must almost always be forward in the sense that many sites within a gene contribute to the rate, rather than just a single one.

Neel: Aren't back mutations a rather special case? Surely in man, and in Drosophila, unless specifically stated to the contrary, the mutation rates are forward mutations. I don't see where this problem of the difference between forward and back mutation has entered into the discussion so far.

Atwood: It comes in in this way, that because it is easy to look for forward mutations in man and easy to look for back mutations in microorganisms, you expect the reported rates to show a discrepancy.

Lederberg: For your purposes, the ranges are not so very different. There are recessive losses of function in the bacteria that have rates anywhere from 10^{-5} to 10^{-11}. Streptomycin resistance happens to be in that category; so there may very well be clusterings to higher frequencies for losses and for returns of function, presumably based on the implicit risk. I don't see how that is going to affect your argument seriously.

Neel: If you could exclude from the bacterial data the back mutation rates, then what would the average be?

Atwood: Only four such values are included.

Zamenhof: But all this applies to spontaneous mutations. Of course, in the case of induced mutations, very frequently, the concept of rate does not apply at all (43). Will that be discussed later?

Atwood: We can discuss that later. Another factor involved here is simply time. If you consider mutation as constant per time unit rather than per cell division then the reported rate, uncorrected for this, would already have about a factor of 10^5 difference, because you have microbial rates being measured in units close to hours, and human rates per generation, about 3×10^5 hours.

Novick: What is the time in the human between successive generations of the spermatogonia?

Atwood: I don't know, but what I meant to say was that the rates are expressed per human generation, not cell generation. In human rate measurements, the individual is assumed to be merely a pair of alleles, and nothing is assumed about cell divisions.

Benzer: But gametes are the result of a large clonal development, during which mutations occur. Is that part of your argument?

Atwood: Yes, that is what I want to get into. Now we can attempt to reconstruct what has gone on between generations, between the time the mutation rate would be measured in one generation and in the next. We have a cell population which will end up as gametes and, for a time, this cell population increases like a culture of microorganisms; that is, with clonal growth. Then it reaches a steady state, in which the population size remains about the same, and you have a stem line regime with the gametes being the overflow.

Auerbach: This is for the male only, this diagram, isn't it?

Atwood: Well, yes; it is probably a little different for the female, since oocytes would not be dividing.

Russell: There is actually a steady drop in numbers in the female, owing to degeneration of oocytes as well as to ovulation. But this probably has no bearing on what you have in mind.

Atwood: In fact, it is even unimportant that there are any females for this argument. If the mutations occur only in males, our estimate based on the entire population would be wrong by only a factor of 2. There is some evidence that human mutation rates are higher in males. This is being argued. Do you want to say anything about it now, Jim?

Neel: I think that Kim is referring to Haldane's 1947 paper (16) on hemophilia. There the basis for the argument is a test for the carrier state in females which is now felt to be highly unreliable. I am afraid that while the methodology in that paper is sound, the laboratory procedures on which Haldane based his arguments are very questionable.

Atwood: It could be done without resorting to a laboratory method of finding carriers, could it not?

Neel: Yes, that is true; but the first approach was, as I stated, based on unreliable laboratory tests (17, 36).

Auerbach: I think, in Drosophila, the males have a higher rate.

Atwood: Yes. Well, in any case, the mutant cells that will be found at reproductive age are accumulating prior to that time. The number of mutants has a different relation to the number of mutations, depending on whether we are in the clonal region of the growth curve or in the steady state region. On the average, the number of mutant cells produced during clonal growth will be greater than the number of mutations by a factor of the log of the number of cells, whereas, in the steady state, the number of mutants and mutations will be the same. We can show this as follows:

Let's imagine that we have N cells at the end of the time, and we start out with N_o cells, which may be the number differentiated off to form this tissue. We divide the time of growth from N_o to N into n intervals. These intervals may be doubling times or any convenient intervals. At the end, the number of cells present is N_oX^n, where n is the number of intervals and X is any base, which would depend for convenience on the interval chosen. You might choose a base of 2 if you wanted to have n doublings. Mutations occur in each interval at a rate, *a,* per cell so that in the first interval we have aN_o mutations but each contributes X^n mutants, because each grows through the n intervals, or a total of aN_oX^n mutants. In the last interval, we would have aN_oX^n mutations, and each contributes 1 mutant. Hence, we again have aN_oX^n mutants.

For any intervening interval, say $n-1$, we have the same relationship; that is, these aN_oX^{n-1} mutations form X mutants each if they are one interval behind, or X^2 mutants per mutation for the second interval behind, and so on. Each generation contributes equally to the final number of mutants, on the average, aN_oX^n mutants. The total final number of mutants is the sum of those contributed for n intervals, naN_oX^n.

If at the end of clonal growth we have 10^{10} germ line cells, as I think would be a reasonable conjecture for the human male, there are about 30 times as many mutant cells at the end than there were mutations that occurred during that period.

Lederberg: That is based on how many generations? How many doublings?

Atwood: Well, 10^{10} is about 33 doublings. Now, thereafter, when

you have the population in a steady state, the number of mutant cells added will correspond just to the number of mutations. Does this require any explanation or is it obvious?

Lederberg: Why number of mutant cells rather than proportion, Kim?

Atwood: Well, the total number of cells stays constant in the steady state, so it's the same thing.

Lederberg: Oh, no! The proportion is always $\frac{M}{N}$, that is, the number of mutant cells over the total number of cells.

Atwood: You are right, it is this ratio.

Lederberg: I see the point now. How do you calculate n? During the chromosomal phase, n is "log" M, but n during the stable phase is some complement of N.

Atwood: Yes.

Goldstein: May I put something in here, because it is going to come up the day after tomorrow again? What we really have to know is the probability that a given cell will be a mutant, so I am not concerned with total numbers of mutants at all. The problem is, given a sperm cell at some time, what is the probability that it will be a mutant? Or, given a cell in a germ line, what is the probability that it will be a mutant? If you make a diagram of a clone, starting with the zygote (Fig. 2), there is a period that you have been talking about in embryonic life when you have this kind of clonal multiplication. Then, there is a period later on when you have a stem cell mechanism, with each division leading to a clone of sperm and to another stem cell.

The point that I would like to make here is that this entire sequence from zygote to sperm, throughout life, is really a single clone. If you look at any clonal diagram, the cells along one edge of the diagram are always sending off subclones. The cells along one edge (and elsewhere) can therefore represent lines of stem cells, as in Figure 2, and the derivative subclones will represent the spermatogonia, spermatocytes, and spermatids of the successive sperm clones derived from that stem line. Consequently, the real difference between the embryonic period and the later one is the difference in interdivision interval that we have been talking about. In the former, the interval is very small; in the stem cell line, presumably, the interval is much greater.

Atwood: When you get a stem line that is mutant, it will simply continue to be mutant, and from then on, every clone that it gives off will produce mutant gametes.

Goldstein: Yes. If you will let me define the probability that I am

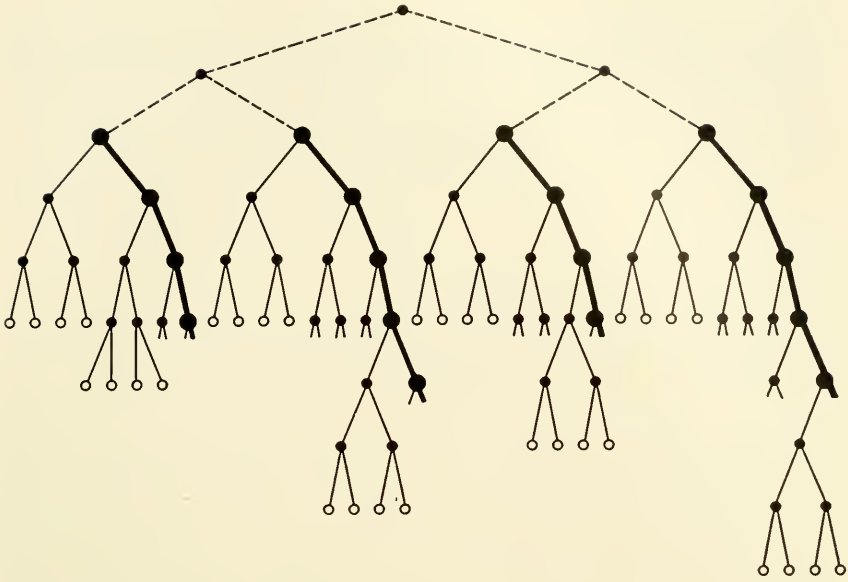

Figure 2. Clone diagram, modified to show stem cell mechanism. The model depicts formation of 4 stem cell lines (heavy lines) each giving rise to subclones (light lines) yielding 4 sperm each (open circles). The diagram emphasizes that the stem cell mechanism is not formally different from ordinary clonal growth. The main differences are (1) that some lines within the clone terminate while others go on, and (2) that the interdivision times may differ between lines within the clone (e.g., the broken lines, light lines, and heavy lines shown here). See also Fig. 25, p. 179.

talking about, it may clarify the thing. Let's take the end result, a sperm, and ask what is the probability that that sperm will be mutant? If we are dealing with a single clone, where all the interdivision times are equal, we could express that probability simply as the probability per cell division times the total number of cell divisions in the origin of that particular sperm.

What I am maintaining is that the only thing that has to be added to this simple formula is that the interdivision time is different in different parts of the clonal diagram. If you knew for any sperm, say at age 30, by how many divisions that sperm was removed from the zygote, and assuming a constant mutation probability per cell cycle, you could easily calculate the probability that this sperm would be mutant.

Steinberg: Shouldn't that equation be modified slightly? The prob-

ability of a mutation occurring in each division is constant and independent of what has gone on before. Then, the probability that the final sperm is a mutation, if there are n divisions before, is one minus the probability that a mutation does not occur, raised to the nth power, in other words, it is a power function and not a straight mutation.

Lederberg: Or the likelihood of double-mutant cells. That's a good approximation.

Steinberg: But this is a probability of a successive nonmutant. It would probably come out the same.

Lederberg: It is a very simple formulation of what I put on the board before. I think I see what you're driving at, Kim, and I think, probably, both of you are quite right. Let's take this formula. It is intuitively obvious that this is the probability that in a given product there is a mutant, and this is the proportion of mutants to totals, in the case where we can take the linear approximation. That is equal to the interval rate times the number of intervals at risk. Now, how do we estimate these? Let me just say, too, you can multiply this out and you get the formula that was given here. During clonal growth, you estimate n as the "log" of N. Later on, there is a period of equilibrium of stem cell production, and then n is proportional to time, and N is not changed proportional to time; so you end up with a formula, if you want to take N into account, the interval of clonal growth—you take the number of intervals that have passed without reference to the increase in the number of cells. This is your old formula.

If you want to write it out in the form you had before, this is for the period of clonal growth. The number of intervals can be counted by seeing how many stems have been produced, and there is a subsequent interval of stem line growth where there is no increase in the number of cells, so we take T period of stem lines, divided by X. That should be a complete description of the probability of the final population of mutants. Is that correct?

Atwood: Yes. I was trying to diagram this situation as you had it, Dr. Goldstein. This is the clone up to the time of establishment of stem cell regime, and this leads to the establishment of a certain number of normal stem lines and a certain number of mutant stem lines. From that time on, every mutant stem line gives off some mutant gametes and every normal one gives off some normal gametes, with the exception of the mutations that may occur in clones intervening between stem cell and gametes.

The mutations occurring in these intervening clones will not show

any age dependence; there will just be a constant proportion of mutants added. But the mutations occurring in the stem lines themselves will give a linear dependence on age, just as mutants accumulate in the chemostat. The stem line is the natural chemotsat. It is a turbidostat, in fact, because we are not clear what is regulating it.

Novick: Is the maturing of a spermatogonium a random process, or is it nonrandom?

Lederberg: It is very nonrandom.

Atwood: Yes.

Lederberg: There has to be another term to this formula, because this takes account only of the mutations in the stem cells. These are the mutations that occur during the growth, the original laying down of the stem cell population. These are mutations that occurred during the subsequent multiplication of the stem cells. But, if you then have an additional element, you are quite right that there are stem cell progeny, which, however, have only limited extent, and once they are formed, they then disappear, but mutations may occur there, so you have to add another term, which is a constant term, which is the size of the progeny, which will be the 4.

Stern: Would you clarify this question of randomness versus nonrandomness of the spermatogonia producing sperm which was just discussed?

Atwood: As I understand it, the question was just how regular is the cell lineage in the production of sperm from gonial cells.

Novick: I thought you told me it was random, that is, the progeny of a given cell might all become mature sperm, and that little line dies out.

Atwood: The way it is usually visualized is that one daughter cell stays as a gonial cell, and the other one goes on to make four sperm.

Lederberg: Yes. The one that stays gonial is the one 2 over X, and then, in addition, you need a term for the addition of sperm from that gonial cell. They have no further future but mutations could occur there, and that is the term, 1 "log" 4.

Goldstein: With advancing age, it is easy to show that the only ones of importance are the mutations in the germ cell line.

Stern: May I ask, Dr. Russell, didn't Oakberg find that this is really much more complicated, that the stem cell divides several times and that some of the resulting cells return to the state of stem cells?

Russell: In the rat, Cleremont and LeBlond (7) estimated that when a stem cell differentiates it produces 64 spermatozoa. Oakberg (26) estimates that, in the mouse, a differentiating stem cell produces,

on the average, approximately 80 spermatozoa. However, I don't believe this alters the basic argument. There are some additional complications; nevertheless, this is still essentially a building-off from one cell that remains.

Atwood: A process of bifurcation of the lines is possible, because that is what must happen if you kill off some cells and they then regenerate. They must make up the number by dividing the lines themselves. That can happen.

Spuhler: Kim, do you have to worry about lines that are killed by the events of mutation? Is it a maximum?

Atwood: They would just be replaced, I surmise. You can regard the system as being the same size, approximately, during the time it is functioning.

Zamenhof: What is the present status of the finding that, in Drosophila, old spermatozoa include more mutations than the young spermatozoa (10)?

Dobzhansky: Detailed data, to my knowledge, have never been published.

Zamenhof: I hope we'll get to a discussion of mutations in stationary cells as compared to where division is going on (32).

Goldstein: Do we know anything about the average interval in the average germ cell line?

Atwood: I don't know. The question of what weights to give to the contributions of mutant gametes from these two periods in the ontogeny of the germ cells is one that we could answer if we knew whether the mutation rate is the same during the one time as it is during the other. If the mutations per division were the same in man, you would get about ten years' worth of mutation or mutant gametes out of the embryonic period.

Neel: You mean that mutationally speaking, the embryonic period makes the same contribution as ten years of adult life?

Atwood: Yes; that is, you would have as great a proportion of mutants at the end of the embryonic period as you would add during ten years of later life.

Novick: I'm sorry to interrupt, but I can only understand this if what Dr. Goldstein says is correct; that is, if the growth rate is different, the generation time is different or the interval is different.

Atwood: The generation time is very different.

Goldstein: What generation time do you assume when you give that figure of ten years?

Atwood: Well, I derived this from the generation time in bone

marrow cells, which is about 100 days. This may or may not be the same for testis.

Magni: Do you assume that the mutation rate is proportional to the number of generations regardless of the generation time in different stages of development?

Atwood: The question is, which should you assume? The estimate I just gave is based on the notion that the mutation rate per cell division is the same whether the cells are dividing rapidly or slowly.

Magni: So, you believe that mutation rate is constant for division and not for absolute time?

Atwood: Yes, that was the case just considered. If you assume, on the other hand, that it is absolute time, then we can ignore the embryonic period, for the mutant accumulation would be linear with time.

Lederberg: Aren't there more direct ways of getting the turnover rate of the testis?

Atwood: One way is by means of a pulse of P^{32} (28).

Lederberg: Are those data known?

Atwood: They are known for the rat and the mouse, but not, to my knowledge, for man (27).

Lederberg: What about the rate of production of spermatozoa divided by the number of spermatocytes? Or the total number of gonial cells in the testis would be more appropriate.

Atwood: You're asking how could you estimate the division rate of the spermatogonial cells from the rate of production of the sperm, as observed, and the number of stem cells present? I'm not sure what the rate of production is.

Lederberg: I suppose these are data that one could get, particularly for man. They must be fairly easily available.

Atwood: Yes. We have considered two alternatives: one, that the rate per division stays the same and, in this case, the mutant accumulation shows a step increase followed by a gradual increase. Then, that the rate per time unit is the same, in which case the early period does not contribute disproportionately to the mutants. We could also suggest that, perhaps, the mutation rate is dependent on the rate of division; in other words, that the mutation rate per division is higher for fast dividing cells than for slow dividing cells. In this case, essentially all the mutants are added at the beginning when the division rate is fast, and then you would notice hardly any effect of age on mutant frequencies since most of the mutant cells would be already there at birth.

If we consider the age dependence of human mutation rates we can

make a preliminary crude guess as to which of these situations applies. This has been considered with two different results. First, in chondrodystrophy, there is a strong dependence on the age of the father. Vogel (42) grouped available data to compare ages of less than 30 with ages of over 35; the difference in mutation rates to dominant chondrodystrophy between these age groups is said to be about a factor of 3.8.

Auerbach: Is this corrected for the age of the mother?

Atwood: Yes. That is hard to unscramble, but it was done.

Demerec: Is that done over a large number of mutations?

Atwood: I don't know; some have argued that dominant chondrodystrophy has more than one locus involved.

Neel: A point here is that of all the achondroplastic infants born, only about 20 per cent survive beyond the first year of life. Vogel's figure is based entirely, as I recall it, on that 20 per cent.

Atwood: The lethal form of it might be a different locus, too.

Neel: Which is not age dependent. Certainly, the age effect in achondroplasia far exceeds that known for such other mutants as neurofibromatosis or retinoblastoma. I don't think we can generalize from this.

Atwood: In any case, if you pick some convenient points, say, age 40 and 25, and assume that these are the mean ages of Vogel's two groups, and then extrapolate back the slope that you get from the factor of 3.8 difference between ages 25 and 40, you find that you come out with a negative value at birth, a very large negative value, which shows that the rate of increase of the mutant gametes with age is high, and not only that, but it shows that the rate is increasing with age.

Auerbach: There could be some discontinuity, couldn't there? Perhaps the chemical environment of the spermatozoa in an old father acts in a mutagenic way.

Atwood: Possibly, in any case, the apparent increase with age is not linear; it accelerates.

Neel: As I recall it, for neurofibromatosis and for aniridia the average father of a presumably mutant child is about a half year older than the population average. I think that would be a little better time interval to work with than the achondroplasia interval.

Atwood: Well, Vogel gives the same or a comparable figure for the things you mentioned; that is, aniridia, neurofibromatosis, and one other, which I don't remember.

Neel: Retinoblastoma?

Atwood: Yes. Now, for these, the factor was 1.26, and if you use

this figure, you get a completely different result on extrapolating back. The result is that you have 30 years' worth of mutants present at birth.

Glass: What is the figure, 1.26?

Atwood: That is the factor by which the apparent rate in fathers over 35 differs from the apparent rate in those under 30.

Lederberg: Is one permitted to say that there is a significant difference in the age dependence of these three conditions?

Atwood: No, I don't think so.

Lederberg: Then there is really not much point in trying to set the models against any one of these alternatives, since they give contrary results, and you can't be sure that the results are different from one another.

Atwood: The data for these three were pooled for the purpose of comparison with achondroplasia. To wind up the question of age dependence, I would say that if, with accumulation of further data, it looks as though there is no significant age dependence, then that suggests that the mutations occur mainly during the prereproductive period, and the stem line mutation rate is too low to measure. On the other hand, if the data are good enough to get the slope of age dependence, then the relative contributions of the clonal and stem line periods can both be estimated.

Lederberg: Can one lump those data? Since they are not heterogeneous, they are homogeneous. You could add them together and arrive at the conclusion that there is an age dependence in mutation rate, implying that mutations are taking place.

Atwood: Some age dependence must exist, unless you are willing to believe that the cell mutation rate during the reproductive period is immeasurably small.

Neel: I think that we can conclude that there is an age dependence effect, but the achondroplasia situation stands out as somewhat different from the other situations in magnitude (25, 29).

Atwood: Yes, it is very different. It is sort of a male counterpart of the situation with mongolism, where we know that the cause is not a mutation of the kind we want to talk about.

Novick: Kim, isn't it the frequency you're speaking about, not the mutation rate? You used the expression that there is a 3.8 times higher mutation rate for the older group than the younger.

Atwood: I meant the relative frequency of mutant gametes. We should understand, though, that this is synonymous with mutation rate as ordinarily expressed in man.

Lederberg: Then, it is a rate per what, if it is a rate?

Atwood: It is a rate per human generation, including all the ages involved. Usually such data are not numerous enough to form age groups, so they are all lumped. In the cases we just talked about, two age groups were formed and compared.

Magni: I want to mention that Cavalli-Sforza (6) in our Department is trying to estimate the age dependence of mutations in man by means of a new method. He is studying the dependence of the sex ratio of the progeny of a woman, on the age of her father at the time of her birth. He has so far data on a sample of about 200,000 Italians and is trying to include the proper question in the census which will be made in Italy in two years. This would yield data on fifty million inhabitants, from which he will be able to have a very precise estimate of the age dependence.

Atwood: Well, if this sort of method shows an age dependence, you might infer something about the sex dependence of the mutation rate. If, for example, the hemophilia rate is related to maternal grandfather's age, then it could be argued that the mutant frequency from females is never higher than the apparent mutant frequency at zero age by extrapolation in males.

Now, I wanted to show what slight evidence I have been able to gather about how we should regard the contribution of embryonic period versus stem cell period. This is with respect to two things, both not conclusive but suggestive of a higher mutation rate in early development. One is the origination of red cell variants with respect to ABO phenotype, and the other is the origination of pigment phenotype sectors in mussel shells.

Magni: Before you enter into details on ABO antigens, Atwood, I would like to mention one point which might be of interest for the previous general discussion.

All that you have said needs an assumption which, for the time being, has not yet been proved true, i.e., that mutation rate per cell per generation is the same in mitosis and meiosis. During the development of germ line in the embryonic period and in the stem line period only mitotic divisions occur, but from the time of sexual maturity, meiotic divisions from the stem line are responsible for the production of mature spermatozoa. If the mutation rate per cell per generation is higher in meiosis than in mitosis, this will certainly affect the apparent over-all mutation frequency.

Lederberg: It would not be dependent on age.

Magni: Well, there will be no clear expectation for age dependence.

We have evidence that, in yeast, mutation rate in meiosis is higher than in mitosis by a factor of ten for the specific mutons analyzed. If this holds true also for man, one should expect two consequences: first, an estimate by excess of the amount of mutations accumulated during the embryonic period and, second, an apparent decrease or even a disappearance of the age effect.

Atwood: If one wanted to complete this notion of different periods, I suppose one would have to separate off the stage just following the embryonic period up to when some sperm are produced, as a sort of static condition in which there is less division than during active gametogenesis.

I want to work up to the present state now, of a study that was begun a few years ago, too optimistically, for the purpose of getting human mutation rates from cells instead of people. The expectations were that if we could find a method of getting the frequencies of mutant cells in individuals, then we could deduce mutation rates from these frequencies and some assumptions about the cell population dynamics.

For this, the ABO locus was chosen, because reagents and methods suggested themselves immediately. I should point out that even if germinal mutation rates at the ABO locus are relatively high, this would be hard to detect. The ABO genotype frequencies are meaningless with respect to determination of mutation rates, because they represent a balanced polymorphic system. We don't know what the selective forces are that keep this system in balance, but they must be potent ones, and capable of operating in different genetic backgrounds. Both man and apes are still polymorphic for the ABO system, despite their other genetic differences.

One could attempt to get such mutation rates from aberrant pedigrees; for example, where an AB mother has an O child, or an O mother has an AB child. These are the only instances in which an ostensible mutation would be generally accepted; other cases in which the identification of the mutation was dependent on the paternity of the child would be equivocal, the mutation rates being lower as a rule than illegitimacy rates.*

We started to look at blood, then, with a method designed to detect low frequencies of non-A or non-B cells in circulating eryth-

* Steinberg estimated the probability that a mutant gamete would find itself in a detectable zygote as:

A or B $\longrightarrow$ O	.02
O $\longrightarrow$ A	.04
O $\longrightarrow$ B	.18

rocytes. These might represent mutant stem cells accumulated in the blood-forming system.

The method depends on agglutination, and this is a reaction that does not go to completion, so in order to get at the frequency of cells that are not agglutinable by an anti-A or anti-B reagent, you can use an iterative process in which you co-precipitate the agglutinable cells with other agglutinable cells that are repeatedly added. To tell the difference between the added cells and the original cells in which you are trying to find the inagglutinable frequency, the original cells are labeled with chromium 51, and the added unlabeled cells act as carrier. We successively agglutinate and separate, until the Cr^{51} activity in the mixture becomes constant (1).

When it has become constant we are not removing any more of the agglutinable cells by addition of further carrier cells in the presence of the agglutinin, so we can say that these cells are, phenotypically at least, not A or B, as the case may be. It should be noted that this phenotypic criterion is operationally valid even if some variant cells can absorb agglutinin but do not agglutinate.

Figure 3 shows a reconstruction experiment. When you add some known inagglutinable cells, O cells in this instance, they stay in the system as the naturally occurring O cells are supposed to stay in it. The system is anti-A with A_1B carrier cells, and a trace of labeled O cells added. Each one of these points is a stage, representing an agglutination and separation. A_1B carrier cells are added to the supernatant at each stage. As we go through these first seven stages, nothing happens to the O cells. They stay in the system; in fact, they seem to increase a little bit.

Now, we add some labeled A_1B cells to increase the Cr^{51} activity about seventyfold and keep on with the same stages. These labeled A_1B that were added are cleaned out rather quickly, as expected, and we come to essentially the same level.

This paradoxical increase in the O cells is real; it comes from the peculiar fact that the volume an agglutinate displaces contains spaces into which the inagglutinable cells cannot find their way; so that every time we discard the agglutinated mass, it actually contains fewer of the added O cells than does an equivalent volume of the supernatant. The supernatant becomes a little more concentrated at each stage. This is corrected for. It may be different with different reagents, so we cannot always expect to come out with an exact level in an experiment unless we have a reconstruction experiment with the same systems, but very often we do come out with an exact level.

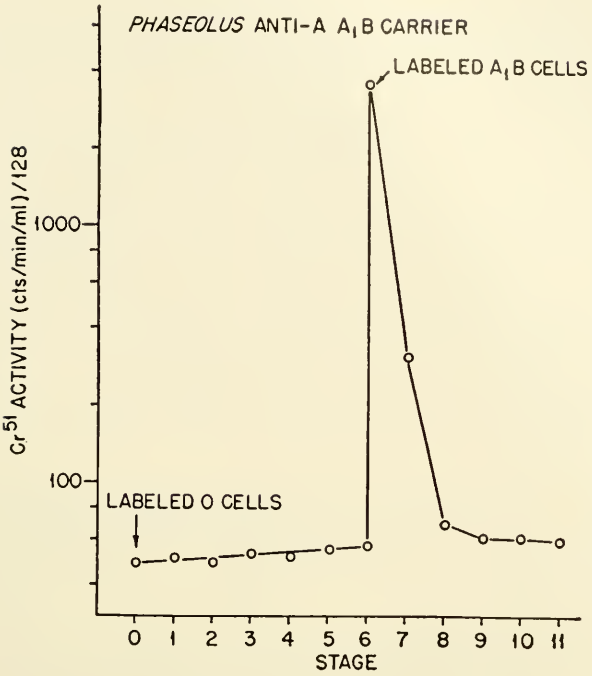

Figure 3. Reconstruction experiment with inagglutinable O cells and agglutinable AB cells. The point on the left is the initial reaction mixture in which only the O cells are labeled. The following six points are supernatants of successive agglutinations with unlabeled AB carrier cells. The high point is the reaction mixture of stage 7 after the addition of labeled AB cells. The remaining five points are supernatants as before. (From Atwood and Scheinberg, *Science* 129: 963-64, 1959, by permission.)

This was done on various people, anyone I could get to volunteer, and we found that A individuals had non-A cells in the amount of something like 1 in 1000. There was a great deal of individual variability.

About two years after that, it became possible to do the same experiments with anti-B, and we obtained the first results from which you could attempt to make sense. We also have an anti-H which, for the purpose of this discussion, I will call anti-O. It agglutinates O cells as

well as A_2 and, to a lesser extent, some others. It is an extract of *Ulex* seeds. Then, we have human anti-A. In the experiments I am about to show human anti-B was one reagent, lima bean anti-A another, and finally the *Ulex* anti-O.

These experiments were designed to test the independence of the losses of A and B in an AB individual. We thought that these might be independent losses, because we were able to isolate non-A cells in sufficient quantity from AB individuals to show that, as a population, those non-A cells were phenotypically B. But we didn't know the exact proportion of B to non-B in the isolated populations. The experiments now are supposed to show what are the proportions of non-A, non-B, and actually of non-O in the same AB individual.

Neel: Of non-O?

Atwood: Yes, there are some non-O cells; that is, they are *Ulex*-negative, at least.

The expectation was that in an AB individual, the product of the non-A by the non-B frequencies should give the frequency of O. This is the expectation if the O cells are formed by independent mutation of the A and B alleles.

Auerbach: That would be a means for ruling out somatic crossing over as a mechanism, then?

Atwood: Somatic crossing over would not affect that expectation in the right direction at all, because what happens with somatic crossing over is that the AB cells are changed into homozygous B and homozygous A cells, which contribute to the non-A and to the non-B frequencies, respectively, and so we get a higher value for their product without having formed any O cells. The AB, AA and BB subpopulations could create O heterozygotes by mutation which again, through somatic recombination, could produce homozygous A's and O's, and so on. As you will see, I have not yet been able to explain the quantitative results by any such scheme.

Auerbach: What I meant was, if the whole effect was due to somatic crossing over, you shouldn't get any O cells.

Atwood: Oh, yes, then the population would move toward an even mixture of A and B.

Lederberg: If this individual were homozygous for the Bombay factor, would you have segregation for O's and A's and B's?

Atwood: Well, I'll give you the result and we can talk about the Bombay factor later.

These experiments are rather difficult to do, which is why I have so few. This experiment (Fig. 4) was done twice, once using the anti-A

first, then the anti-B, and then the *Ulex*, and, a second time, using the anti-A first and then the anti-B and the *Ulex*. This first curve represents the proportion of the activity remaining in cells versus the number of stages with anti-A and A carrier. This is an AB individual to start with. We see that he has 7×10^{-3} non-A cells.

Now, we continue the same experiment and remove a very large proportion of the remaining cells with anti-B and B carrier, and finally a further removal with *Ulex* and O carrier. Then, the reverse experiment is done. With the anti-B first, he has 10^{-3} non-B cells. Most of these are A. When both of the reagents are used in tandem, of course, you have to come out at the same level whichever the order of removal. It does make that much sense, at least.

When we use *Ulex* to remove the cells that are ostensibly O at the end of this experiment, we remove some, and the difference between

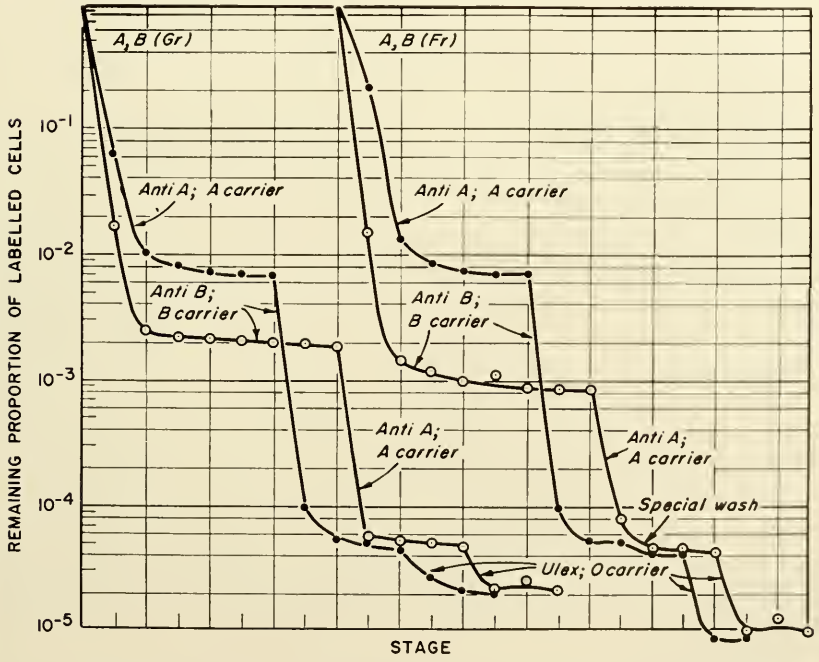

Figure 4. Isotope dilution curves showing phenotypic subpopulations in two A_1B bloods. The criterion of cell phenotype is coagglutination with the agglutinin and its corresponding carrier cells. Phaseolus anti-A, human anti-B and *Ulex* anti-H are used in sequence: solid points= anti-A first; open points=anti-B first.

these two levels is the proportion of O cells. This difference is 3×10^{-5}. The expectation for 7×10^{-3} and 2×10^{-3} is 1.4×10^{-5}; so that the O cells are in excess by a factor of about 2.

Figure 4 shows much the same situation. This is another individual. With anti-A—this is purely fortuitous—he has the same non-A level as the other one. There is a lot of variability. Most people actually have a non-A level of 10^{-3}.

Well, then, adding anti-B and B carrier, we sweep out a lot of remaining cells, and then, with *Ulex,* we sweep out still more of the remaining cells. The same when anti-B is used first. In this case, the non-B level is 2×10^{-3}, and then, with anti-A, we can sweep out most of the remaining ones and we come to the same level here. The little jogs appearing from time to time in these curves represent times when we got worried about activity in the supernatant that was not in cells, because of lysis that goes on during the experiment. This requires a special wash. We always suspect such activity after a certain number of stages, and rightly so. We get rid of the label in solution by washing the cell samples in a very large volume of saline before the final centrifugation and Cr^{51} counting, so that we are dealing only with activity in cells and not in solution.

Here, the expectation is 7×10^{-6}, and what we observed was 4×10^{-5} of O cells. You have, in this case, an even greater excess of O cells than in the other experiment.

The question of the identity of the cells that are not A, B, or O, but are left here after *Ulex,* is perfectly open. A possible explanation for them is that they are Bombay cells, as Dr. Lederberg suggested; at least they are not agglutinable by *Ulex,* and that is a characteristic of Bombay cells.

Lederberg: Are they erythrocytes?

Atwood: Yes, they are probably erythrocytes, because they are lyzable. That's all we know about them.

Lederberg: What would that rule out?

Atwood: It would rule out white cells, generally speaking. You lyse in 2 per cent acetic and this would save the label in the white cells, and release the label from red cells. The same proportion of the label can be freed from sedimentability at the end of the experiment as at the beginning.

As to other particulates anywhere above this bottom level you would have to assume they behave like red cells toward these agglutinins, so I am not worried about them too much.

Cotterman: I'm not sure how this question comes into the argument

and the calculations, but if you sediment and lyse, you're going to produce ghosts. I'm curious to know just what strength of centrifugation you used. Would it have been sufficient to throw down the ghost cells?

Atwood: Yes, it would, and if you lyse chromium-labeled cells, the ghosts contain from 15 to 20 per cent of the label. The remainder goes into the supernatant and is mostly in the hemoglobin, probably because the chromium binds generally to protein, and hemoglobin is the main protein present.

Lederberg: Do ghosts behave like the intact red cells in their agglutination behavior?

Cotterman: Yes, they do.

Neel: Kim, I seem to recall that in other work where you presumably were using a homozygous A individual, the proportion of exceptional cells was higher than computed on the basis of two independent events. Is that not correct?

Atwood: Yes, that is correct, but it is not certain whether that individual was homozygous A, although it is highly probable. Also, the experiment was not carried enough stages to get a real level. It began to hook over at about 10^{-4}, so probably the level would not have been much below that. Comparable A heterozygotes have about 10^{-3}, so there is a tremendous discrepancy between the expected (10^{-6}) and the observed (ca. 10^{-4}) if that person is a homozygote. We now have two known B homozygotes, and they show just this kind of discrepancy.

Before talking about these I want to mention two attempts to find changes other than losses, that is, whether any B cells are produced in A individuals. Experiments of this kind would not ordinarily be meaningful because the B cells originating in an O or an A individual would not have a normal life span. The isoagglutinin would destroy the circulating cells, and perhaps also the exceptional stem cells.

However, we had a chance to see whether A goes to B in two agammaglobulinemics who had no demonstrable anti-B, although they were both A's. Judging from their non-A frequencies, they may be heterozygous A. I say this with some reservation, because I am not yet sure how often we would be wrong with this criterion.

Cotterman: You had no family data on these two individuals?

Atwood: Yes, but it is inconclusive. After the experiments, we tested the survival of B cells in one of these agammaglobulinemics by injection of 2 ml. of Cr^{51} labeled cells. The survival of the injected B cells

was normal, so we concluded that if such cells had been produced, they would have persisted.

We had two opportunities to study these. The first one was a test for A to B, consisting of cleaning out all the A cells with anti-A and then starting with anti-B and seeing whether any further cells were removed with anti-B and B carrier, and finally putting some *Ulex* in, to be sure that the cells remaining were actually O.

This experiment (Fig. 5) was done by Takashi Ito in some very care-

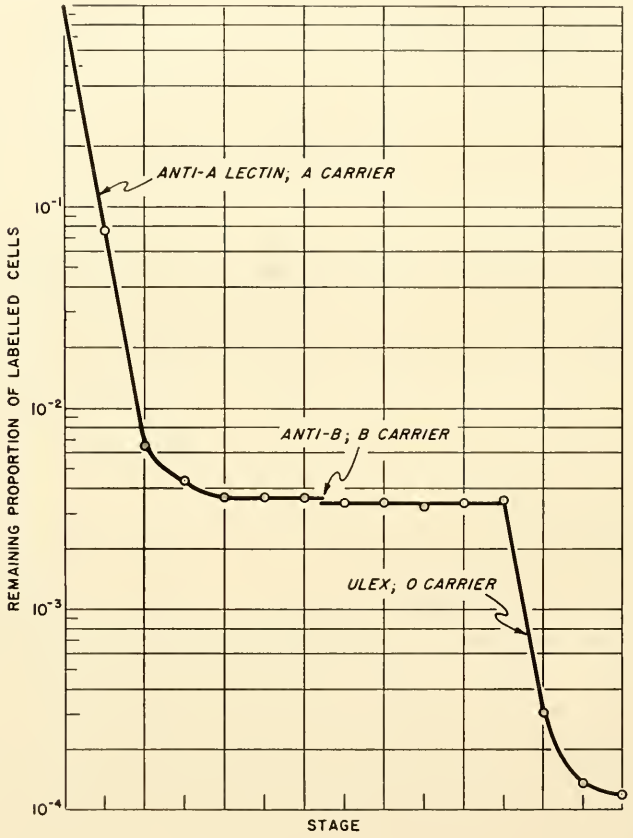

Figure 5. Isotope dilution curve for an agammaglobulinemic, group A, probable heterozygote. Anti-A, anti-B, and *Ulex* in sequence. Absence of B cells is suggested by minimal effect of anti-B. The curve is broken at this point to indicate the size of the possible B cell population, but its reality is questionable.

ful work. This is the isotope dilution curve with anti-A and A carrier. At this point, anti-B and B carrier regime is started. Whether anything happens is a matter of conjecture. I have plotted it in such a way as to emphasize that something might have happened; that is, you might have a little bit of decrement at the beginning here with anti-B and B carrier.

After five stages of this, *Ulex* and O carrier are added, and there is a large removal of cells. If any B cells are present in this individual, they can't be more than 10 per cent of the remaining non-A cells, whereas the O cells amount to nearly all of the remaining cells. You can't say that no B cells are produced from A, but you can say that if they are produced, they are produced much less frequently than O cells are produced from A.

This is consistent with the notion that there are more ways of changing the gene into a form not making A than into a form that makes B.

Goodgal: Can you add the *Ulex* first and then measure what is left?

Atwood: That can be done, and should have been. Actually, however, we did an experiment in which anti-B was added first.

If we have a heterozygote, which we can designate as AO and we are getting some A to B, then this would give you OB cells that would be detected in here. But if we have O going to B, it gives you an AB cell, which is removed here (upper part of curve) and therefore is not detectable.

In a futile attempt to look for O to B, we ran some stages with anti-B at the beginning, before removing cells with anti-A. This approach is very insensitive, though (Fig. 6).

This second individual had a lower proportion of non-A cells than the first. This curve is with anti-A alone. The other curve has three preliminary stages of anti-B which, at the same rate of removal as in other experiments, should have brought any B cells down to a vanishingly low proportion. No removal was detected. Then, the anti-A is run, and you see that it is hard to decide whether the difference between the two curves is real. No real difference is expected in any case.

Ito did some experiments with normal people who had the isoagglutinins as well, and got the same sort of equivocal results with them as with the first case, so I should guess that essentially no mutations that create B cells are occurring.

Glass: We have some data that would bear out this conclusion, from growing various kinds of tissue cells in culture and then testing them

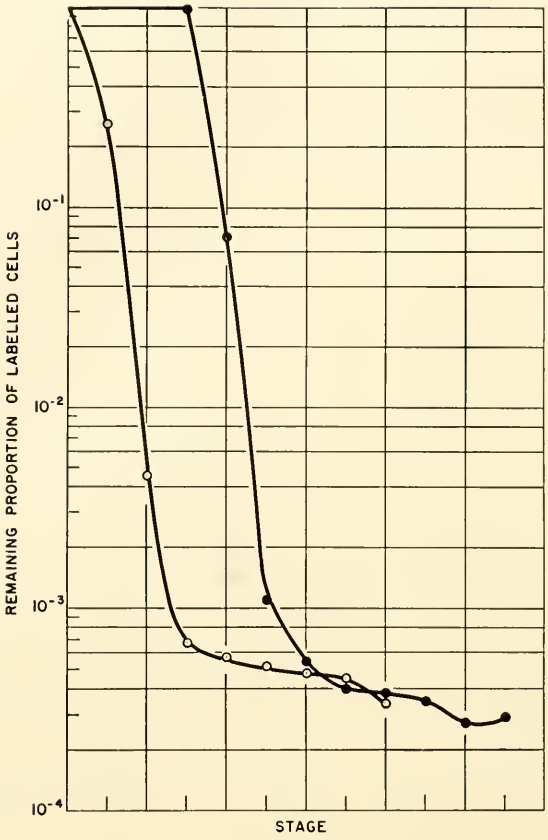

Figure 6. Isotope dilution curves for a second agammaglobulinemic, group A. Open points = anti-A alone; solid points = anti-A (following three stages with anti-B and B carrier).

with the mixed coagglutination test. We felt that the most critical kind of test would be to use O cells and then look in control cultures that had not been treated or in cultures that had been X-rayed for cells that gave a type-B reaction when B red cells and anti-B serum were added. We have never gotten any indication of a change from O to B.

Lederberg: What about fluorescent antibody?

Glass: We don't have any data on this.

Atwood: Until recently I had thought that fluorescent antibody could

not be used with red cells. The amount bound by red cells is less than that of tissue cells, so you haven't as much fluorescence to see around a red cell. But such techniques have been worked out. I can't remember the reference, but now it should be possible to use fluorescent antibody, and that is a much better way than these indirect ways that I have just given, provided you are looking for positive cells in a negative background.

Neel: This is work by Wolf Zuelzer and Flossie Cohen in Detroit (8).

Cotterman: Were they using a fluorescein-labeled anti-A or anti-B or fluorescein-labeled anti-antibody, or both?

Neel: I can't recall.

Cotterman: That last possibility I mentioned is one to keep in mind; if your tagged fluorescent antibody does not give sufficient fluorescence, you can, perhaps, double up on this by using an anti-antibody and have both of them labeled with your fluorescent dye.

Neel: Would an antibody work after an anti-antibody had doubled up on it?

Cotterman: Yes. This technique has been used in other fields.

Atwood: One might suppose from the lack of independence of the two alleles that mutation may be playing a minor role, and that many exceptional cells are phenocopies that represent simply a failure to form the antigen, even though the stem cell progenitor was of normal genotype.

Another type of evidence on the question of how the cells originate is even more confusing than what I have just shown. It is the effect of radiation on the process. We studied two polycythemics who were going to be treated with P^{32}, with the idea of killing off part of their bone marrow so they would no longer form too many red cells. We got blood from them just before the P^{32} injection and followed their exceptional cell levels at intervals afterward. Before going further, I must refer to the A subgroups.

The A phenotype has certain variants, A_1, A_2 and weaker subgroups. But the principal distinction here is that if you take ordinary human isoanti-A and absorb it with certain A's, you find that there will be activity left against certain other A's. On the other hand, if you take those other A's and absorb the serum no activity is left, so we can subdivide A's according to whether they remove all activity from isoantibody or only part of it.

If they remove all the activity, they are called A_1; if they remove part of it, they are called A_2, or some other subgroup of A that is

determined by additional criteria. We have a specific anti-A_1, the extract of Dolichos beans, and so, in an individual that is A_1, it was possible to find out how many of the cells are phenotypically A_2, as well as how many are phenotypically O.

Incidentally, it was possible to find out, in an A_1B, what proportion of cells were A_2B, as well as just B, and this was done with the idea of telling whether somatic crossing over or other processes that give homozygosis is the main origin of exceptional cells. The answer was that A_2B cells were present. They are not homozygotes. Homozygosis is therefore not the only thing involved; we must also have some change from A_1 to A_2.

Some have argued that A_2 may be a quantitative change in the agglutinogen rather than a real difference in specificity. I prefer the notion that it has different specificity.

In any case, in these two polycythemics we followed both the non-A_1 and the total non-A cells. We did the regular isotope dilution experiments with both Dolichos anti-A_1 and lima bean anti-A.

The first one did this (Fig. 7). This is time (abscissa), and each one of these points is the level that we obtained with an isotope dilution experiment, this array of points being the non-A_1 with Dolichos (Fig. 8, A.R.), and this (Fig. 7, A.R.) being simply non-A.

Notice that with lima bean anti-A, after the initial changes you are left with a stable increase 170 days later. There is another point, not on this figure, which is about a year later. It is only a little below this last point. This, then, is some sort of stable change. You can ask, how does this change compare with stability of the levels in normal individuals? We have only a few data on this. I would say that it is much more stable in the normal individuals than in those who had the P^{32}. You have only a little variation over periods of three years, and that variation is technical error, I am convinced. This is not technical error.

Now, what happened here (Fig. 7)? This may be partly technical error, because we found the specificity of the Dolichos is influenced a great deal by the temperature at which the experiments are done. This is not true of the lima bean. Therefore, with summer coming on here, we may have an artifact in this curve.

Auerbach: Excuse me, but what does this original rise signify?

Atwood: What does it suggest? Figure 8 is another case, and we see just a rise of the total non-A (Fig. 7, G.P.). On the other hand, we see this early spiking of the assumed non-A_1 (Fig. 8, G.P.). I wouldn't think too much about the reliability of this kind of spiking, unless it

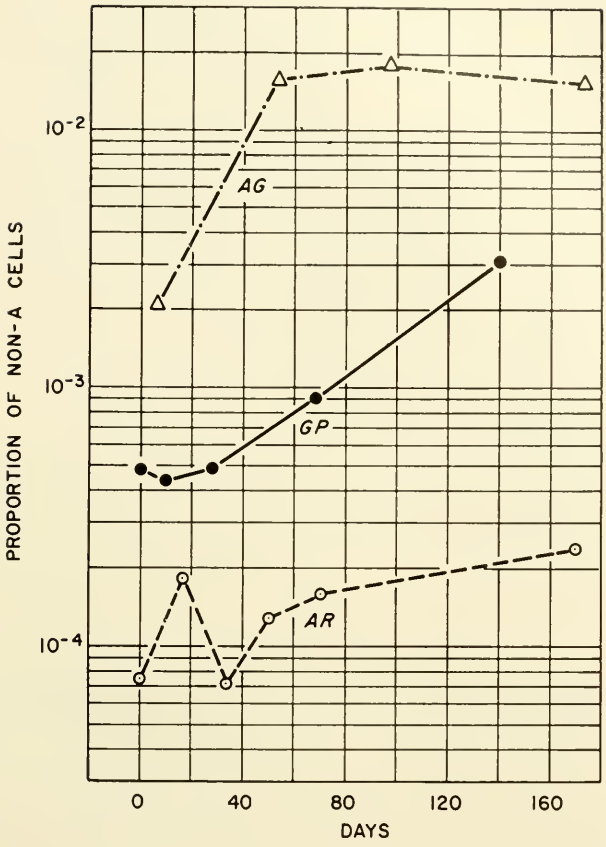

Figure 7. Proportion of Phaseolus-negative cells in polycythemics treated with P^{32}. A.G. is the A_2 subject reported by Scheinberg and Reckel (*Science* 131: 1887-1888, 1960); the first point is seven days posttreatment with 2 mc. of P^{32}. G.P. and A.R. are A_1, treated with 4 mc. of P^{32} on day zero. An additional point was obtained for A.R. 424 days posttreatment: 1.5×10^{-4}.

were so very large. This is more than a twentyfold difference between here and there. There must be something going on that is real.

Scheinberg, who worked with me on this at Oak Ridge, is working with a pigeon system of blood groups that can be used for these experiments. The positive pigeons have erythrocytes agglutinable by lima

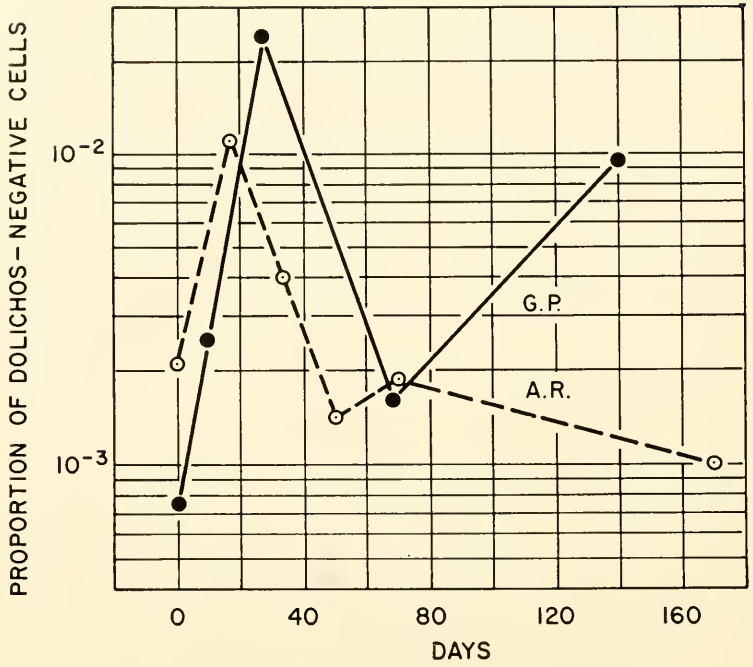

Figure 8. Proportions of Dolichos-negative cells in subjects G.P. and A.R.

bean extract, and the negative ones inagglutinable. He could give the pigeons wholebody X-ray, and when he did that, he got the same kinds of results as shown in this figure: initial increases falling down to a stable level, above the preirradiation level in all his cases.

I don't have his graphs here, but I can show what some of the levels were. He recently published in *Science,* and you may have seen these data (Table 1) and some additional ones (33, 34). The pigeons, by and large, have higher frequencies than people do, if you use pigeon cell carrier. You can also use human A cell carrier. Then, they have frequencies more or less similar to people. But in any case, no matter which carrier is used, a long time afterward—this is 220 days, 430 days, 206 days, and so forth—you have large stable increases in the negative cells in basically Phaseolus-positive pigeons. These increases are much greater than you would anticipate on the basis of specific locus induced mutation rate, and that is why they are so confusing.

TABLE 1

Effect of irradiation on the frequency of inagglutinable cells. Dose is expressed in roentgens to the peritoneum. The inagglutinable frequencies are listed as F and the days following irradiation as D. (From S. L. Scheinberg and R. P. Reckel, *Science* 131: 1887-1888, (1960), by permission.)

Subject number	Dose	Initial F	D	F	D	F	D	F	D	F
					Pigeon cell carrier, X-rays					
930	0	0.0100	33	0.0090	34	0.0098				
954	500	0.1080	40	0.3370	109	0.2150	204	0.2000	432	0.2411
2943	630	0.0460	51	0.3550	126	0.2520	221	0.1700		
986	745	0.0076			104	0.0360	208	0.0128		
921	818	0.0810	40	0.1250						
					Human A cell carrier, X-rays					
893	832	0.0011	54	0.0021						
					Human A cell carrier, γ rays from 4π cobalt source					
951	101	0.0028	48	0.0026	149	0.0025				
926	500	0.0014	46	0.0180	120	0.0042	192	0.0050		
908	1004	0.0010	49	0.0030			243	0.0030*		

* *P. lunatus* extract prepared at later date than extract used initially.

When you knock out the bone marrow with radiation it has to go through a period of clonal growth to catch up again. This offers an opportunity for selection in which the negative cells might win out. That could explain these radiation results, as well as the ones in man. On the other hand, if we assume the same selection factor for O cells in heterozygous and homozygous A individuals, for example, then mutation and selection would produce an even greater difference between the O proportions in the heterozygote and homozygote than would mutation alone. But we actually observe less difference. This kind of selection would not give you an excess of the kind observed.

You would think that, if selection is involved, the starting number of the negative cells would be related rather directly to the amount of increase with radiation, whereas, if mutation alone is involved, there would be no relation between them. The starting number is there, and with a certain dose of radiation you produce so many mutant lines. This number would be quite independent of how many negative cells are present at the start. More experiments are needed before this point is clarified and they should be done all with the same kind of carrier. With pigeon cell carrier, you have anywhere from 10 to 100 times as many negative cells as you do with human carrier.

Neel: In the pigeons, are the differences between individuals before treatment greater than have been observed in man?

Atwood: They are, if you use pigeon cell carrier. When I learned of the carrier effect it scared me because I thought, well, everything is artefact now. We have to use blood bank sources of carrier, and we don't know anything about them. We tried different carriers on the same blood and never found any carrier effect with man at all. This restores a measure of confidence.

The carrier effect could be explained on the assumption that there is a specific difference between the pigeon and human cells and that part of the lectin is bi-specific or heteroligating. This would explain a phenomenon that Scheinberg observed in following up the effect, namely, that if he isolates a negative population of cells from a positive pigeon by successive sedimentation with lectin, and washes the cells that are not agglutinable by lectin, then as soon as he adds human A_1 cells, the pigeon erythrocytes accumulate a rosette of human erythrocytes. This suggests that they were coated with antibody but not agglutinated, and that the basis of the carrier effect is that, with pigeon cell carrier, you reach a point where there are a few cells left that are coated with a bi-specific lectin, the pigeon end of which is inside and the human end outside, and cells coated in this way can attach only to human cells and cannot stick to other pigeon cells.

Cotterman: I would say that this idea of what you call bi-specific antibody is quite tenable. I have had some evidence of this in studies of these bi-agglutinins on different animal species, and I believe it is something you've got to find.

Atwood: Scheinberg is not convinced that this idea is right. He thinks it has something to do with the site number. By that, I mean the number of agglutinogen sites per cell, which he assumes is greater for human cells than for pigeon cells. Consequently, if this residuum of isolated pigeon cells is a fraction having the lowest site number, so that it is inagglutinable because of insufficient cell to cell binding, then, if human cells are smaller and have more sites they might become attached.

Lederberg: There is nothing here that says that the two ends of the individual lectin have to have different qualitative specificities.

Atwood: No, not in Scheinberg's explanation.

Freese: Isn't it possible that the initial rise after the P^{32} treatment is due to the incorporation of the P^{32} into RNA, the subsequent decay preventing the formation of A-antigen? After some time, new RNA is made and the antigen can be produced again.

Atwood: Yes, or if you say that it kills certain specific RNA molecular species—

Freese: No, it need not be specific.

Lederberg: It may be some of the RNA in some of the cells.

Atwood: The reason I would reject the idea at first is that this implies that you would have killed the cell by a number of other means as well. If you have enough P^{32} activity in the RNA to prevent the antigen synthesis, the cell probably would not mature since other syntheses would also be blocked. But I see this is not necessarily right. A certain proportion of the cells would escape all the other damage and have only the antigen knocked out. Perhaps we are seeing a small enough proportion to be accounted for in that way.

Freese: There may be much more incorporation into RNA than into DNA; thus transient effects may be more pronounced than killing.

Lederberg: Much of this depends on the timing. When is the antigen synthesized in the developing erythrocyte?

Atwood: We don't know for man, although I think it could be found out quickly. In the pigeon, the bone marrow cells of both white and red cell series are equally agglutinable by Phaseolus as the peripheral red cells, so there is no difference (34). The phenotype runs throughout in the pigeon.

Lederberg: It is probably not true of human leukocytes, is it?

Atwood: I don't know.

Lederberg: Which would, if so, already constitute a difference. You must know about that, Bentley.

Glass: We have never been able to demonstrate any antigens on white cells.

Atwood: By fluorescent technique or anything else?

Glass: We haven't used fluorescent methods.

Cotterman: You mean by agglutination?

Glass: Mixed eo-agglutination tests.

Cotterman: What type of reagent did you use?

Glass: Anti-A, anti-B, and anti-H.

Cotterman: Phytoagglutinins, as well as sera?

Glass. Kodani did use *Ulex* agglutinins (19).

Lederberg: These, presumably, would be the lymphocytes and polymorphs, but not the monocytes. Monocytes certainly do have surface antigen, don't they?

Glass: I don't believe that Kodani was able to make a distinction between types of white cells.

Auerbach: Wasn't this rise also observed for the A_1, which is still antigenic?

Atwood: Yes. It is not stable for the A_2.

Auerbach: But could it not be some phenocopy effect? About a year ago, there was an article in *Nature* (14), in which somebody working with leukemic patients found that during the leukemic attack he got A_2 in an A_1 patient. It went away when the man was treated. When he had a recurrence, the same change in the blood group took place. Might the introduction of the P^{32} have a similar effect?

Atwood: We could speak of the penetrance of the gene at the cell level and say that it has 99.9 per cent penetrance, which is why we see 1 in 1000 non-A cells. This is a terrrible idea, but it might be true.

Lederberg: Why terrible?

Atwood: The actual value of penetrance now has to be explained. Why should it be of the order of 1 in 1000 or 1 in 10,000?

Neel: Kim, aren't you the person who suggested variation in the number of reactive sites on the red cell as an explanation of non-penetrance?

Atwood: Yes, that you might have a distribution of sites that is characteristic of that site system. Cells in the tail end of the distribution could have too few sites to be agglutinable.

Lederberg: Can you relate the time of the P^{32} decay with the time of appearance of these aberrant cells? Just when in the history of a

given red cell has the P^{32} decay taken place in relation to when you see it in the aberrant cell?

Atwood: I have been told that nearly all the P^{32} effect is accomplished in the first week after injection.

Lederberg: If you put in effect a pulse, then how long after that do you first see the aberrant cells?

Atwood: About two weeks.

Lederberg: I mean, at what stage of development were these cells at the time the P^{32} was put in?

Atwood: Probably near the end, because they appeared in two weeks or less. I don't know how much less, but in about two weeks there was an elevation of the exceptional cells.

Lederberg: So it is not unreasonable that this is a time of determination of the surface antigen, either with disintegration in RNA or DNA, for that matter. You may be getting chromosome breakage effects such as Pardee was talking about (21).

Atwood: Well, chromosome breakage is a leading hypothesis. It would explain it perfectly if we had not done the experiments with the Dolichos.

Lederberg: No, that's not out, either, because you had a cell that was just starting to make A_1, and A_2 was then the stage in the amount of the production of A_1.

Atwood: Yes, that would explain it; that amounts to a hypothesis about the formation of the antigen. I don't know whether or not A_1 is made by finishing off an A_2.

Lederberg: Well, A_2 may be a lot of A_1, or conversely, A_1 may be a lot of A_2, but in either case you could get this result by doing a complete job of manufacture.

Auerbach: Wouldn't you then need selection against them, because this peak disappeared?

Lederberg: Well, these are the cells, and this is a phenotypic effect. This is not taking place in the stem line, you see.

Atwood: The nice thing about chromosome breakage is that you could imagine that the cell with chromosome losses might still be able to mature from within the clone leading to red cells, but not be able to survive in the stem line.

Neel: Isn't there a complication in the quantitative treatment of this problem that has not been mentioned?

Atwood: What is that?

Neel: The maximum anemia in a polycythemic following treat-

ment with P^{32} does not occur until—what is it—two months after therapy.

Atwood: Yes. The reason for that is the lifetime of the peripheral cell.

Neel: Precisely. That's what I was getting at. So the pulse has to be viewed in relation to the total red cell count for a quantitative treatment.

Lederberg: The pulse that we are speaking of may not be influencing the production of fully chromic, euchromic erythrocytes; that is, you may be impairing the capacity to form surface antigen in a fraction of cells, but not impairing the production of hemoglobin in most of the cells.

Atwood: Yes, I see.

Lederberg: Only later on have you hit those cells that were starting to make the hemoglobin at the time you put in the pulse of P^{32}. Then, your anemia comes in.

Atwood: You remember, this peak came down again well within the lifetime of the peripheral erythrocyte, which is about 100 or 120 days. This means that the kind of cell that makes up this peak must have a short lifetime.

Lederberg: Or else it is finishing up the job. Perhaps these cells are now maturing; in other words, what you are seeing here is delayed maturation rather than interrupted maturation.

Atwood. You mean delayed until after they are in the peripheral circulation?

Lederberg: Yes.

Atwood: This would be unexpected.

Lederberg: Yes. Do you know that they are not reticulocytes?

Atwood: No, you're right; they may be reticulocytes.

Cotterman: I might say that I looked at the proportion of reticulocytes in the so-called A_2 + O mosaics which I studied, and found no unusual proportion.

Atwood: Yours were very frank mosaics, indeed, though, weren't they?

Cotterman: Yes.

Atwood: You would suppose that with the processes of mutation, when we have stem line regime and so on, you would get a marked age dependence, and so, about two years ago, we took all the isotope dilution experiments that had been done up to that time and plotted them against the ages of the individuals. Unfortunately, we did not have a very good spread of ages, especially among the young.

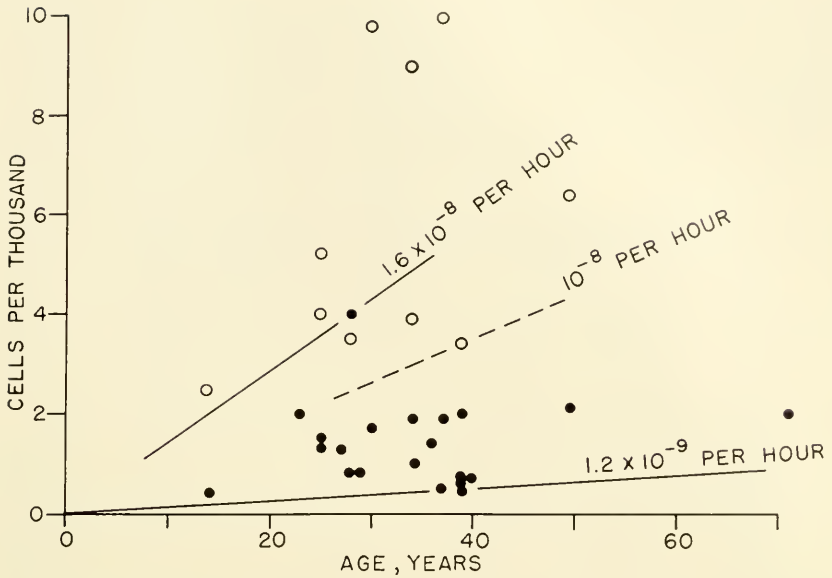

Figure 9. Proportions of Phaseolus-negative (solid points) and Dolichos-negative (open points) cells *vs.* age in A and AB persons. Lines show required rates of origination of exceptional stem lines on the assumption of linear increase from age zero. Subsequent data (Figs. 10-12) show this assumption to be erroneous.

The black points on Figure 9 are the total non-A and the open circles are the non-A_1—the non-A_1 includes phenotypically A_2 and O cells, summed. The slopes represent the rates of mutation necessary to produce these observed frequencies on the assumption of linear increase with age.

To produce the lowest frequency would have required about 1.6×10^{-9} mutations per cell per hour; the highest (for non-A) about 10^{-8} per cell per hour. These open points are too high to be considered as mutant frequencies.

As you will see, I think the assumption of linear increase from the origin is false. The rates estimated on this false assumption would not be unusual by microbial standards, but are about a factor of 10 above the usual range of human germinal rates.

These scattered points do not suggest an age effect, but it is not ruled out. The different individuals may have had different rates of accumulation of mutant stem lines. Even if this were true, very young

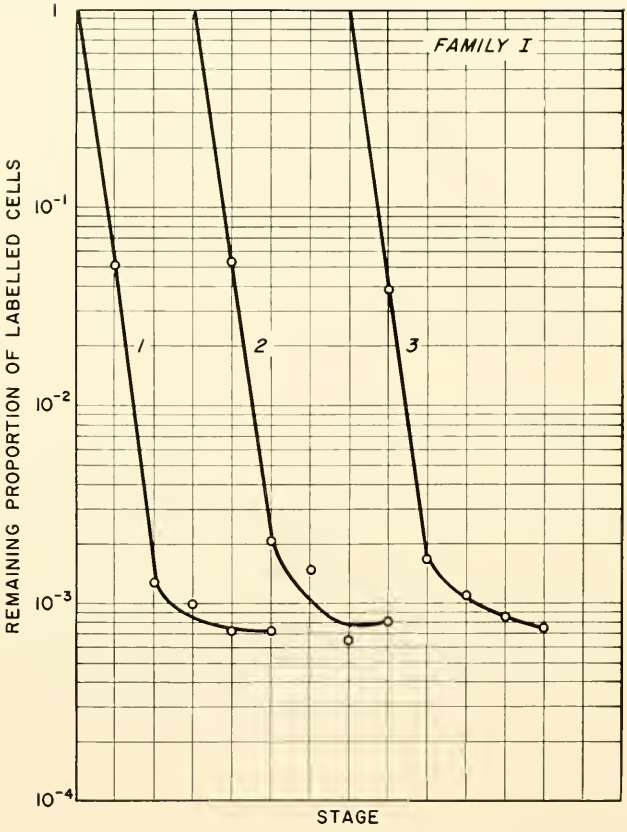

Figure 10. Isotope dilution curves with anti-B for male parent, A_2B (curve 1); female parent A_1B (curve 2); 11 month male infant, A_2B (curve 3).

individuals would generally have low frequencies of mutant cells. We have a few experiments on parents and children of known genotypes.

In this experiment (Fig. 10), which is with anti-B, the parents are both AB. One is an A_1B, and one is an A_2B. The infant, 11 months old, is an A_2B. You can see that the non-B proportions are identical. The B allele of the infant is one of the parental B alleles, and the age differential is large. This was done through the Chicago Blood Donors Association. They were very cooperative in trying to find families with AB parents.

Cotterman: How was the blood-type determination made on the child? With Dolichos anti-A_1?

Atwood: Yes, Dolichos anti-A_1. You mean, to know if it was an A_2B?

Cotterman: Yes; if there is some difficulty in subtyping, you know, with certain reagents. I haven't studied an 11-month-old infant, but on anti-A_1, while Dolichos is an excellent reagent for an adult, it does not work well at all on newborns.

Auerbach: Are these estimated rates high enough that if you retested a person in, say, five years, you should find a difference, even on the lower estimate of mutation frequency?

Atwood: In ten years you could. The first experiments were done about three and a half years ago. But I think that the few family studies we now have strongly suggest that there is not going to be much effect of age.

Lederberg: You mean, there is a strong age dependence, but your technique is completely unable to show it because of the scatter of individuals?

Atwood: No, I surmise it does not show because such a large proportion of exceptional cells is already present in infancy.

Figure 11 shows a family in which both parents are AB, one is A_1B, and one is some subgroup of A. I don't want to call it A_2 necessarily, because of the following: when we took the parent who was originally grouped as A_2B, and two children, one aged 4 and one aged 22 months, and did the isotope-dilution experiment, we got the results shown in this inset. Each of them has about 30 per cent of inagglutinables as might have been expected from the appearance of the slide test. This is the kind of mosaic that you have worked on, Dr. Cotterman.

Cotterman: Yes. Did you say that one parent was A_2B and the child seemed to be something that you might want to call even weaker than an A_2B?

Atwood: No, in this instance they are alike; perhaps they should be called A_3B. The mosaicism of this particular phenotype is already manifest in the young child. This is the parent who was an A_1B; she had 6×10^{-4} non-A cells. These (A_3B) individuals had about 30 per cent of non-A cells, and these are two other members of the sibship. One of them is a twin of one up here, a dizygotic twin, of course. Here is the pedigree. This pair of twins is an A_1A_3 and A_3B, and they are four years old. This other A_3B is about two years old, and this A_1A_3 is five years old.

These are two A_1A_3's and they have levels which are just about the right amount less than that of the A_1B parent so that it would be safe to say that the mosaicism associated with this subgroup is quite independent of the loss of A_1. In other words, the subpopulations that

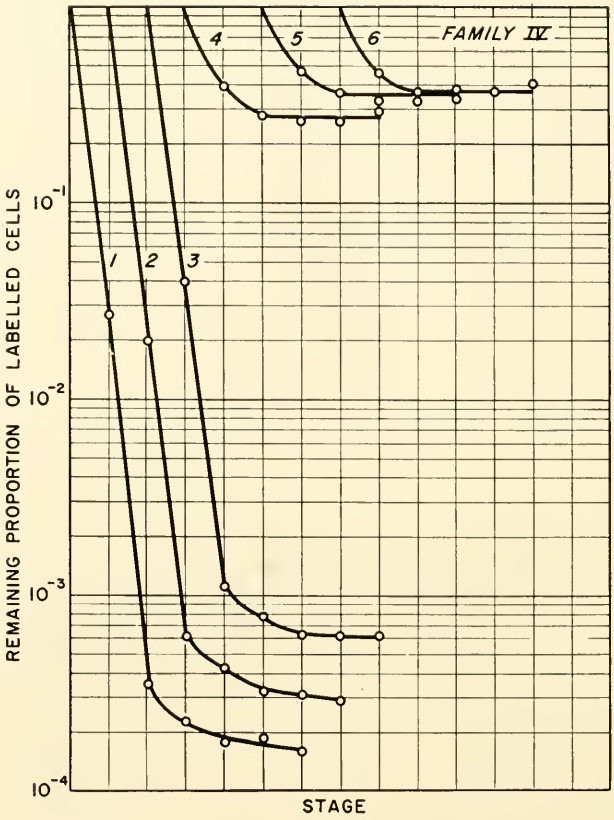

Figure 11. Isotope dilution curves with (human) anti-A

Key:	curve	subject	sex	age (yrs.)	genotype
	1	D.S.	F	5	A_1A_3
	2	M.S.*	M	4	A_1A_3
	3	P.S.	F	25	A_1B
	4	L.S.*	F	4	A_3B
	5	R.S.	M	2	A_3B
	6	W.S.	M	28	A_3B

* M.S. and L.S. are dizygotic twins.

have lost A_1 in these A_1A_3 cases probably have a mosaic phenotype equivalent to that in the A_3B relatives. I say probably, only because there is some individual variation in loss of A_1.

Cotterman: Did your A_1B parent show a greater than average proportion of non-A cells?

Atwood: No; 6×10^{-4} is a little less than the usual for A_1B.

Freese: What is the number of stem cells in the bone marrow?

Atwood: About 10^{11}, I think, or maybe a little more than that.

Goldstein: What is the average clone size from one stem cell?

Atwood: If you assume the number of stem cells to be about 10^{11}, then the clone size would be about 100, because of the order of 10^{13} erythrocytes in an individual.

Dobzhansky: Could you make clear for me what you mean by "mosaic"? Do you mean that this individual has two different genotypes in different tissues, or do you simply mean that phenotypically the blood cells have a different count?

Atwood: Phenotypically, the cell population appears mixed. What the origin of such mosaics may be, I don't know, but they are associated with the subgroup called A_3. There is a disagreement about whether A_3 is really a mosaic of A_2 and O cells as Dr. Cotterman has suggested.

Neel: In the mosaics, what are the proportions of the two types of cells?

Atwood: About 70 per cent are A_2B, apparently, and about 30 per cent are apparently just B, although it has been claimed that these inagglutinable cells adsorb anti-A.

I infer from the isotope dilution experiments that the A_3 allele in the A_1A_3 individuals is also giving a mosaic phenotype when this is revealed through loss of A_1.

Cotterman: Would you put down the proportions of the other phenotypes for the A_1A_3?

Atwood: Where is that graph? This individual (A_1B) has 6×10^{-4}. Now, the A_1A_3's, those are the ones you want?

Lederberg: This is the frequency of non-A cells, is that right?

Atwood: Yes, in this A_1A_3 individual. It's 3×10^{-4}. This one (A_1A_3) is 1.6×10^{-4}.

Neel: Non-A, not non-A_1?

Atwood: Yes, not just non-A_1. If you want to be strictly operational about it, it is the frequency of cells that are not coprecipitated with A_1 carrier in the presence of unabsorbed human isoantibody that is anti-A. These experiments, by the way, unlike the others, were done with human anti-A rather than lectin.

Auerbach: It's 30 per cent of 6.

Atwood: Yes.

Auerbach: If this is a very weak antigenic form, then it looks more like a question of nonpenetrance, 30 per cent nonpenetrance.

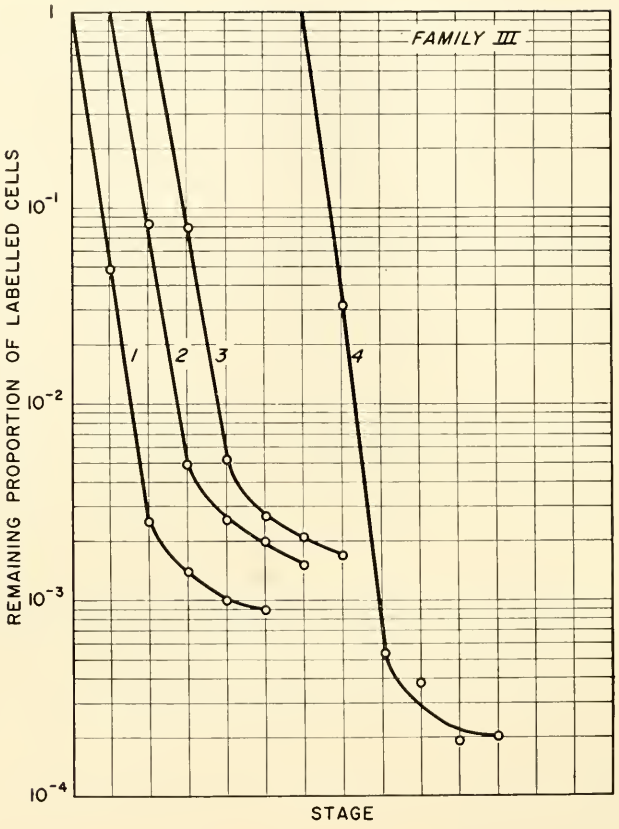

Figure 12. Isotope dilution curves with anti-B

Key: curve	subject	sex	age (yrs.)	genotype
1	Ke.F.	M	10	A_1B
2	A.F.	F	32	A_1B
3	W.F.	M	36	A_1B
4	Ka.F.	M	12	BB

Atwood: Oh, yes, 70 per cent penetrance of the A_2, if you look at it that way.

Glass: But you have the same A_2 allele in the ones that are mosaic and in the ones that are not mosaic.

Atwood: You see, these (A_1A_3) can't be mosaics in the sense that these (A_3B) are because they have an A_1, so most of their cells are going to be phenotypically A_1, anyway.

Cotterman: Did you find three kinds of cells that you could classify as A_1, A_2, and O?

Atwood: In these individuals, no. We didn't use any agglutinin except human anti-A, which will agglutinate both the A_2 and the A_1.

Cotterman: You would say that almost all the cells are A_1 or A_2 and only a small fraction are O?

Atwood: Yes, that's right.

Cotterman: But you can't say what fraction of the cells among the A's are phenotype A_1 or A_2? You didn't test them?

Atwood: No, we didn't have enough blood.

Well, now, finally, I have this discouraging news. Among these families with both parents AB, we had two B homozygotes, and so were able to compare homozygotes and heterozygotes with respect to frequency of non-B.

The first instance here in Figure 12 again demonstrates absence of age effect. This experiment is technically not so good as the other because you are still removing some cells at the end of the experiment, but it is approximately completed. The parents have levels of about 1.5×10^{-3} of non-B cells, and one child has about 10^{-3} of non-B cells. He is the AB child. The B homozygote has 2×10^{-4}. We have never found an AB that had that few non-B cells. Even so, the observed frequency is much too high to represent the product of the frequencies of losses of the individual B alleles. The expected frequency is around 10^{-6} on the assumption of independent behavior of the alleles.

Lederberg: What is the product of the non-O there?

Atwood: We didn't have an opportunity to continue these experiments with *Ulex* and O carrier.

Lederberg: What was the value with *Ulex* in the previous blood?

Atwood: In the previous (AB) blood, the *Ulex* following anti-A and anti-B removed all but about 10^{-5}.

Lederberg: Another factor of 10, then?

Atwood: Yes, or nearly so.

In Figure 13 the parents are both A_1B. Their non-B frequencies are different; one has about 10^{-3} and the other about 2×10^{-3}. One of the children has about 10^{-3} and the other about 2×10^{-3}. You might think, therefore, that the non-B frequency is a part of the phenotype of the given B allele, as though this one got the B allele from this parent, and this one got the B allele from that parent. I surmise that this is just chance, however.

The non-A frequencies were also done on these; they are the same for all four of the individuals, 10^{-3}.

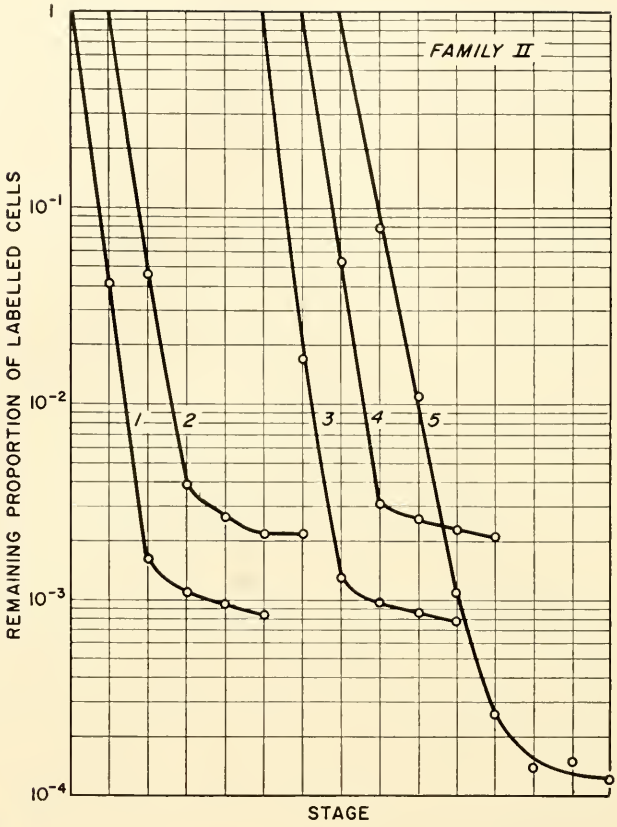

Figure 13. Isotope dilution curves with anti-B

Key:	curve	subject	sex	age	genotype
	1	I.O.	M	49	A_1B
	2	H.O.	F	47	A_1B
	3	Wa.O.	M	21	A_1B
	4	Br.O.	M	8	A_1B
	5	De.O.	M	14	BB

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The B homozygote in this instance has about 10^{-4}. Again, it is the same sort of discrepancy; that is, he has from 10 to 20 times fewer non-B cells than the A_1B's, when he ought to have about 1000 times fewer.

Stern: May I ask a question? Both you and Dr. Cotterman said that this A_1 is associated with another B allele. Is it not sufficient to

say that it is associated with another genotype? Can you say, with these few samples, whether it is the other allele or anything else in the genotype, which makes for the change? It's just equivalent, isn't it?

Atwood: It is for the few cases I have been discussing.

Cotterman: But we have this, Dr. Stern, in quite a few individuals in the family, and over two or three generations. This has been observed in so many pedigrees that it would be unlikely that the modifications were due to many other genes than the B allele itself.

Lederberg: That's fine. Is there any example of an interaction at all? What Kim called the A_2 allele is simply expressing itself in only 70 per cent of the cells in which it appears, regardless of what the other genes are doing independently.

Atwood: That is what this indicates. We have no particular reason to think it is expressing itself differently in A_1A_3's than in A_3B's. It seems to express itself in just the same way.

Lederberg: The A_1 covers up the A_2. It's only when the A_1 is dropped out that—

Atwood: Exactly, which would bring you down to about the level of 6×10^{-4}. Then, you get a slightly lower level, because the A_3 isn't completely expressed.

Goldstein: One of the problems that arises from what you have been telling us, it seems to me, which relates to the question of age dependence or non-age dependence, is the question whether the changes that you have been discussing arise from the stem cell line or whether they arise during the clone multiplication from stem cells.

Atwood: That is certainly correct.

Goldstein: It seems to me that it should be possible to regard each clone of final erythrocytes as an independent population, with an average life of 120 days. If one made repeated determinations, say, at six-month intervals in the same individual, one should have, in essence, a variance test. Well, one should expect, then, high variance between such determinations if these were arising primarily during the clonal development of erythrocytes and not primarily if they were in the stem cell line. Would that be a possible thing to do? Do you have any data on the consistency of repeated determinations in a single individual over a long period of time?

Atwood: It is stable over three years.

Now, your question as to the variance introduced by mutations occurring in the intervening clone between the stem line and the peripheral erythrocyte has an answer that depends on the number of such clones whose products are in the circulation at the same instant.

I should say that this number must be very large. If you have, for example, 10^{11} stem cells and 10^{13} erythrocytes, and clone size intervening is about 100, then you have the products of all these 10^{11} clones in the circulation; even with large individual clonal variances, no variation from time to time would be observed. Does that answer your question?

Goldstein: Yes.

Atwood: I want to consider now what assumptions one would have to make to still preserve the notion that the exceptional cells found in people by isotope-dilution methods are products of mutational events taking place at various times in the erythropoietic tissue.

First of all, since we have essentially no age effect, we would have to make the assumption that the main population of mutant stem lines is established very early. We could also ask whether the rate of mutation per cell division during this early period is higher than it is later. As you recall, we estimated the number of divisions preceding the steady state condition to be about the same as you have in ten years during the steady state. If the mutation rate per division is the same at all times, about ten years' worth of mutants would be established during the early period.

If the mutation rate per division is itself dependent on the division rate, which is much higher in the early period, then a still higher proportion of the mutants would be established early, and that is what the limited data we now have seem to suggest.

The second thing is that in order to regard the exceptional red cell data as meaningful in this context, the lack of independence of the alleles would have to be explained.

I want to discuss both of these points in sequence. The first one is the reason for bringing up the shell mosaics in mussels.

I happened to notice, while eating some mussels (*Mytilus edulis*), that the population was polymorphic. In certain populations, some individuals are unable to form the blue pigment that is normally in the shell. Something less than 1 per cent (I don't know the frequency) of mussels in the area of Woods Hole, Massachusetts, are deficient in pigment, but about 60 per cent of these have one or more blue sectors in the shell that could be interpreted as reversions to the ability to form pigment.

The umbo is the point of origin of the shell. One sees concentric growth rings from which one can deduce the size and shape that the animal must have had in the past, and the revertant sectors cut across these, from the umbo to the periphery. The shell-forming gland is a

strip of tissue running around the edge. It is obvious from the relations of these structures that in order to form a pigmented sector the gland must have a little island of cells in it here that are producing pigmented shell, whereas the rest are producing nonpigmented shell.

Then, you might infer that if there is no differential growth rate of the cells that are producing pigment as compared with those that are not, the time of origin of a sector from a single cell could be deduced simply by measuring the width of the sector out here where it is wide enough to measure, and measuring the perimeter of the shell. The ratio of the sector width to the perimeter immediately gives the number of cells in the gland at the time of origin. For example, if this is 1 per cent of the perimeter, then we had 100 cells present when this sector originated as a single cell.

Auerbach: Why do they always go back to the umbo?

Atwood: When the mussel first sits down after its free-swimming larval stage, this represents the point at which the earliest shell is formed, and from this point it grows, so now, if you establish early a little island of cells that form pigmented tissue and they maintain their spatial relation to the other cells, this sector has to come out from the origin.

Dobzhansky: Does the sector always reach the umbo?

Atwood: The shell-forming gland is a columnar epithelium that has cells 4 micra in width, so that one has to accumulate a fair number of these cells before the sector is big enough to see. Therefore, the sectors seem to vanish as they recede to the umbo. But we know that the sector really must originate from somewhere down here. If a sector were to originate out here where the animal is large and then, let's say, the perimeter doubles, you would now have on the average two cells instead of one. You would never detect it. Therefore, any sectors originating far out are going to be undetectable until a lot of growth occurs. But awareness of this fact has enabled the deductions that I am about to make, from having measured such sectors.

You see, the reason for doing this was because it seemed to afford an analogous situation to the absence of age effect, as you will see. If we were to take this shell and grind it up and measure how much pigment was in it, this would be analogous to taking blood and seeing what proportion of cells are exceptional. However, instead of that, we have a permanent record of everything that happened in this shell, up to a certain size.

The number of sectors per shell is better correlated between right and left halves of the same animal than among different animals, but

the positions of the sectors in right and left halves of the same animal are independent.

Lederberg: What is the source of the shell-to-shell variance, which is responsible for the apparent correlation?

Atwood: Simply that these two halves of the same animal have the same rates of sector origination.

Lederberg: But then different shells have different rates of origination.

Atwood: That's right; different individuals do have different rates of origination.

Auerbach: Why do you think they should have different rates between animals?

Atwood: We have no idea what the genetic basis for the sectoring is or why it has different rates.

Cotterman: You're talking now about the small minority of shells that show some variegation. Are the rest all uniformly blue?

Atwood: Most of them are uniformly blue, but there is another class that is blue, basically, but with a few colorless sectors. These are hard to discover because the shell gland, except in very small individuals, is thick enough so that an island of nonpigment-forming cells in it will make a white sector imbedded in blue. This is much harder to see than the reverse situation. I don't know the frequency of these except that it is higher than the frequency of basically albinos with a few blue sectors. Some individuals are not easy to place in either category.

Occasionally, you find one that has thousands of very narrow sectors that all seem to originate at about the same time. I am not considering these in the present discussion.

Lederberg: In view of the variation from individual to individual, are you going to question the regularity of the cell lineages, and the number of stripes you may get, which may simply reflect how dispersed the cells from a single mutant clone may be? There is no certainty that different stripes come from really independent clones.

Atwood: You mean that when there are several sectors, these may have had a common origin, but somehow the cells became dispersed.

The point I'm going to get at is that the rate of origination is high early, and declines later. A dispersal such as you suggest would mean that the true originations were even earlier than it would appear from any of the resultant sectors.

Auerbach: But if you can't discover the ones which happened late, these might have happened more frequently—

Atwood: That problem has a simple solution; we can find the rates

of origination, relative to cell number, for sectors that are all well within the visible range. I threw out about 30 per cent of the visible sectors as being too small to measure. I am purposely taking a certain range of sizes and finding out within that range what is the distribution.

Benzer: The variegation has been studied in detail in corn and shown to have this kind of basis.

Atwood: What kind of basis?

Benzer: Early mutations arising at different times along the development of the cell line.

Atwood: I am asking the question, is the rate of origination constant per cell, or not, as the animal grows. The answer is that in this case it is not. You can plot now the number of cells at time of origin versus the (per cell) frequency of origins corresponding to such numbers. It looks like this (Fig. 14) in the particular ones that I measured; 420 sectors.

Benzer: What is that curve?

Novick: He dates the things by the number of stripes he sees. This is the number having a given date.

Benzer: Number per time?

Novick: Size of the stripe; so that most, or the majority, of the stripes originate at about 100 cells, and by the time there are 1000 cells, there are almost no more origins.

Lederberg: What is the evil of picking them up, though, by the entire scale? Don't you have some difficulty in seeing them?

Atwood: There are a lot more beyond 1000 that I excluded purposely to show that these can all be picked up.

Lederberg: Do you know that you can pick them up if the stripe is 1000?

Atwood: I know there are some more here than I can't pick up. But what you are saying is how do I know this rate of origination won't go up again sometime in the future, when all are too small to see?

Lederberg: The curve is naturally flat, but you have less and less chance of seeing the events that occur at those stages, because they result in—

Atwood: But, up to here and even past here, they are all visible. I measured the widths up to here only. If a sector were so narrow that it was invisible, it would be out here somewhere. It would have a number of cells at time of origin greater than 1000.

Lederberg: You don't think there are any other stripes as wide as the ones that you have recorded that you have failed to record because they were not contrasting enough?

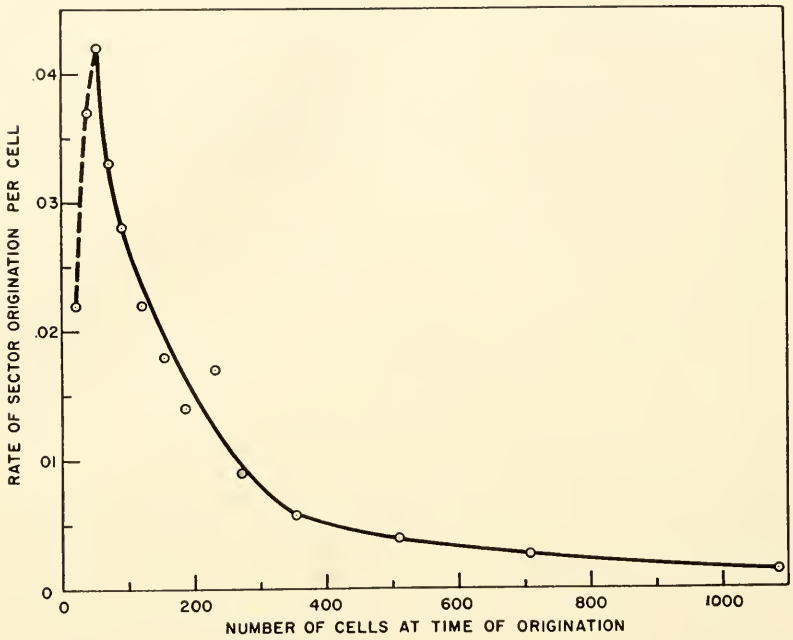

Figure 14. Sector origination rate per cell *vs.* cell number in the shell-forming gland of Mytilus. The ratios of perimeters to sector widths for 420 sectors were arranged in rank order, and groups of 30 were formed. The difference between the lowest and highest ratio in a group is an estimate of the number of cells in which the 30 sectors originated. These estimates were converted to sectors per cell and plotted *vs.* the median ratios of the corresponding groups, that is, the median cell number for each 30 sectors. The dotted line region may show an artifact caused by compound sectors of common origin.

Atwood: I don't think so. I feel very strongly about that point.

Auerbach: But couldn't there be some kind of competition in growing out, which would discriminate against the narrower ones?

Atwood: There could be, but generally speaking, there is not, because if you look at the cases where you have the two sectors very close together, then the increment of width of the intervening white part and that of the sector itself is similar. We have not any special evidence of differential growth rate, at least not for most of them.

Of course, these are really approximations because, as the shell grows, it changes shape, so there is a rubber-sheet distortion. Certain parts get disproportionately wider and others do not. But this does

not make more than a factor of 2 difference, and doesn't change this picture at all.

This decline prior to 100 cells, I think, is an artifact. An explanation of the decline is that many of the sectors are noticeably compound—just the phenomenon you referred to, Dr. Lederberg. They obviously have the same origin, but they consist of several lines very close together. Wherever possible, those lines were measured separately, but if they had been summed, they would have brought the number of very wide sectors, which are the ones in here (very early), to be higher, and might have brought them up to 4 per cent.

This may not be the complete explanation, but the compound sectors are actually more frequent than the simple ones.

Dobzhansky: What proportion of the albino shells show these lines, anyway?

Atwood: About 60 per cent of them. The distribution of lines is such that the genetic basis is most likely heterogeneous; the rates are so different among individuals.

Glass: This phenomenon reminds me very strongly of the sectorials analyzed by Brink in maize (3).

Atwood: Yes, that's right. I merely put this forth to show that at least here is an example where some sort of genetic change—and I say "genetic" only in the sense that the cells inherit pigment forming ability from their progenitors—has a rather restricted time of occurrence. Perhaps it will be possible to learn the genetic basis through breeding experiments.

Neel: Can Dr. Demerec clarify for us how comparable this is to the plant situation?

Demerec: It seems to me that the kind of behavior described by Atwood is similar to that found in plants, particularly corn, where the growth patterns of leaf tissue and seed pericarp resemble the growth pattern of the mussel shell. Many years ago, I did a considerable amount of work on just this type of genetic change, studying unstable genes in the larkspur, *Delphinium ajacis,* and in *Drosophila virilis* (9). In Delphinium two flower-color genes were studied in detail, namely, rose and lavender. Both mutate to the dominant allele which is purple, and the mutant cells show up as purple sectors on a rose or lavender background. Since the color is limited to one layer of epidermal cells in the sepals and petals, and the cells are large enough to be seen under a low-power microscope, it was possible to determine the size of the purple sectors in terms of the number of cells they contained. The purple sectors on the sepals were counted and classified into

categories ranging from 1 to 4,096 purple cells, each category having double the number of cells of the preceding one. It was assumed that, on the average, a mutation responsible for a sector having twice as many cells as another sector had occurred one cell generation earlier. Theoretically, each category represented mutations that had occurred during the same cell generation. One-cell sectors originated through mutations in the last cell division, two-cell sectors originated in the division before the last, and 4,096 sectors in the twelfth division before the last. Mutations occurring in the early stages of the development of a plant showed up as chimeras with a purple sector extending over several flowers. The data revealed that the rose gene mutated at the same rate throughout twelve divisions of the epidermal cells of the sepals, and that the mutation rate was similar in the earlier stages of the ontogeny of the plant. Depending on the strain, the rate was between 0.22 and 8.1 per 10^4 cells per one cell division. The lavender gene, on the other hand, had a high rate of mutability early and late in ontogeny, and during the intermediate stages the rate was very low or the gene remained stable. These results make it seem probable that mutations occur during the multiplication of genes, and, since one-celled sectors were present, they support the hypothesis that gene multiplication is accomplished by the formation of a new gene, by the old one, rather than division of the gene into two equal parts.

Of the three unstable genes studied in *Drosophila virilis,* reddish-alpha mutates at the reduction division of heterozygous females only; magenta-alpha and miniature-alpha mutate in the germ cells and in the somatic tissue only; and miniature-beta does not revert to the wild type at all, but changes with a low frequency to the more mutable gamma or alpha forms. "It appears as if a mutable gene becomes unstable whenever the cell reaches a certain stage in the development of the organism where, probably, the chemical properties of the cytoplasm are such that they stimulate the reversions in the genes. Different genes, apparently, require different conditions to become mutable" (9).

Auerbach: I was just going to ask how general you think this is, because in McClintock's cases, you may get what you call a change in state, which changes simply the time of origin of the sectors.

Atwood: I don't know how general this phenomenon is. This is an example of something that happens in a certain time range, rather early. There are other examples of genetic changes that happen over different time ranges. In this instance the cell division rate during the maximum time of origination is higher than at any later time, so if the process turns out to be mutation, then we would say that this mutation

rate per cell division is actually higher when the cells are dividing rapidly than when they are dividing slowly. The mature mussel grows about a centimeter in length in a year, so that in a year it does not even double the perimeter, if it is already of fair size. This means that, barring turnover, the shell gland cells in large specimens divide less than once a year.

Freese: I think that you have to be very careful about the word "mutation" in this connection, because you cannot even exclude cytoplasmic inheritance, in which cytoplasmic particles segregate early in the life of the mussel.

Atwood: Yes, the genetic basis isn't known.

Now we come to the problem of how the two alleles at the ABO locus do not seem to be independent. Independence of mutational events has seldom been tested. Perhaps others here can cite such examples. One was that, many years ago, Szybalski and Bryson compared single and double mutations in *E. coli* (39). The double mutations, if I remember correctly, were at least ten times more frequent than the product of the single frequencies, or perhaps more than that. Does anyone recall that paper?

Demerec: Yes, the material has been published only in abstract form, in the annual reports of the Biological Laboratory (4, 5). He was observing not reversions but changes from the wild type to the mutant type.

Magni: We recently had some evidence on an effect similar to the one mentioned by Dr. Atwood. We have studied back mutation induced by X-rays for two independent mutons in the same methionine cistron in yeast. A diploid strain was used which was carrying the two mutants in transposition, and because they were not complementing each other, it was methionine dependent. After X-ray treatment methionine independent colonies were selected and genetically analyzed. The preliminary result was that, with an X-ray dose inducing back mutations at any one of the two sites with a frequency of the order of 10^{-3}, we found double mutants with a frequency of 2-3 $\times$ 10^{-5} while the expected one was 10^{-6}.

Freese: Was it hard or soft X-ray?

Magni: It was very soft. The water half-value-layer was 2 mm.

Auerbach: Did you ever see whether you got something more comparable to Dr. Atwood's story? Did you ever find both alleles of the same locus mutate simultaneously in the same cell?

Magni: I have no data on true alleles. The mutants I have used can be more properly considered as pseudoalleles.

Auerbach: But they were in opposite chromosome?

Magni: Yes.

Atwood: Among the explanations that were suggested for Szybalski and Bryson's case was the possibility that only a small proportion of the cells accounts for a majority of the mutation, and this leads immediately to an apparent lack of independence. Is it clear why it should, or should I give a numerical example?

Novick: Yes, do.

Atwood: Let's say that the apparent rates of the two are 10^{-7}, so then you expect the double mutation to be 10^{-14}. But let's say that, as an extreme case, 1 per cent of the cells are mutable and 99 per cent are not, so that the actual rate accounting for just the mutable cells is 10^{-5}, you see.

Now, among this 1 per cent of cells, the double mutants will be 10^{-10}, and since they are occurring only in 1 per cent of the cells, the observed double mutant frequency relative to the total population would be 10^{-12}, so in this case you expect 10^{-14}, and you obtain a hundred times more than that. That kind of explanation is a suitable hypothesis for further investigation.

Zamenhof: We have found one case of a double mutant (11) which was peculiar. The mutants were obtained stepwise; that is, it is not the case where only one gene is involved; the parent strain was tryptophan-dependent which was also induced to be histidine-dependent by heat. This double histidine-dependent tryptophan-dependent mutates back to histidine-independent tryptophan-independent every time by a single mutational step. Moreover, by transformation we always obtained histidine-independent tryptophan-independent by a single transformation. Thus, one "back mutation" affects two functions, tryptophan function and histidine function. To repeat, the mutants were obtained by two separate mutational steps, but they mutate back in a single event, and transformation always occurs with what we call 100 per cent linkage, that is, the cell is always transformed to both prototrophic markers together.

Freese: What is your evidence for two different cistrons?

Zamenhof: The tryptophan locus is known to influence one enzyme, and histidine locus, another one.

Freese: This you assume.

Zamenhof: In this particular case, we know it.

Freese: I mean, it could be that one genetic region would be involved in both enzymes.

Zamenhof: One region which is necessary for both enzymes?

Novick: I am reminded of the very famous case of Zinder and Lederberg (46), where they were finally the same locus.

Lederberg: No, I don't think so. They simply were closely linked. I might say that we have been studying a system very much like what Dr. Zamenhof has mentioned, in *B. subtilis* involving tryptophan and histidine markers. They are behaving perfectly conventionally in our case, not necessarily the same mutants (37). They revert and conform conventionally.*

Zamenhof: We have three linkage cases (11). In two of them, we have only 65 per cent linkage. But this one is 100 per cent.

Auerbach: When you have them reverted, can you make them mutate individually again?

Zamenhof: We didn't try that. But they were obtained individually.

Auerbach: Yes, but how could you see whether they were still there as an individual mutable unit? Will you now try to get auxotrophs again?

Zamenhof: We haven't tried yet.

Atwood: I want to close by saying that I think we have enough reason to suppose that the excessive human mutation rates, as compared with mutation rates in microorganisms do not necessarily involve major differences in the frequencies of the individual microevents that constitute mutation.

The purpose to be served by a mutation rate has more to do with its validity than the value itself in this sense: if you are interested in the effect of mutation on human gene frequencies, then the rates as estimated in man are appropriate, but if you are interested in understanding the mutation process, these rates are likely to be misleading and the rates measured by better methods in microorganisms are more appropriate.

Auerbach: May I ask one question? It is really connected with what Dr. Magni said. For years, I have been trying to find the evidence for the statement that mutation does occur independently in two allelomorphs in the same nucleus. Muller, for instance, writes that when you have a nucleus, one gene mutates, and there is no more than a random chance that the allelomorph on the opposite chromosome will also mutate. This is not easy to establish, and I don't really know what the evidence is.

Stern: Formerly, the evidence was seen in the following facts. In Drosophila, a female which is heterozygous for a normal and a white allele has red eyes. If a mutation occurs from normal to white, a white

* Sneath and Lederberg, 1961.

spot will be formed in the eye on the red background. Such spots occur with a certain frequency. In a fly which is homozygous for the normal allele white spots will be formed only when both alleles have mutated. The extremely low frequency of white spots in homozygous red eyes, assuming that they occur at all, seemed to indicate that simultaneous mutations of both alleles are much less frequent than mutation of a single allele. The argument, however, has been greatly weakened by the finding that white spots in heterozygotes may not be the result of mutation but of somatic crossing over.*

Auerbach: But I wonder if there is any other evidence for it.

Magni: We have data in one case which is perhaps not perfectly pertinent but nevertheless is worthwhile to be mentioned. Studying the spontaneous back mutation rate of site met_{1-1} in yeast we have observed a discrepancy between mutation rate in haploid strains and in diploids homozygous for the same mutant, the rate in diploids being higher than expected.

Auerbach: It should be done in the same cell.

Stern: There is similar evidence in Drosophila derived from the frequency of white spots in hemizygous and homozygous red-eyed individuals. In hemizygotes the spots are more frequent than supposedly in homozygotes. Here, somatic crossing over is, of course, ruled out as an explanation, but as in yeast the difference in mutation rates is obtained in different types of cells, those with one and those with two alleles.

Lederberg: I think there are any number of cases in pericarp and in the aleurone mutation in corn where it has been established that the mutation goes from a homozygous to a heterozygous condition, by examining the embryo in the rows. I don't think it can be a question of the possibility of qualitative independence of mutation in one allele and another. I don't know of any case outside of the microorganisms, either, which would make any quantitative statements as to the frequency of mutation. I remember looking quite hard for this a while ago.

Auerbach: But it could be checked in plants, really, as to the mutation frequency.

Lederberg: I don't know where in plants you really have that good a measurement of rate of mutation, outside of the somatic cell situation, and then there is always this question.

* Addendum: The problem of somatic mutation of one or both alleles has first been discussed by R. A. Emerson. The nature of bud variations as indicated by their mode of inheritance, *Am. Nat.* 56: 64-79 (1922). He concluded that only one of two alleles underwent mutation.

Stern: In the snapdragon, *Antirrhinum,* there are some reports on simultaneous mutation of two alleles (38).

Auerbach: Spontaneous?

Stern: Yes. Some of these cases concern highly mutable genes.

Auerbach: Because in induced mutation, there are several people who make the statement that chromosome breaks (which, of course, are something different) tend to occur in homologous spots in the two chromosomes, in genetic treatment.

Neel: Kim, if I understand correctly, your message is that in mutation, no matter how we measure it and no matter in what form, the important thing is the cell division; that if we put rates in terms of cell divisions, our mutation rates will be very similar from one form to another. The apparently higher rate in man is related to the greater number of cell divisions that intervene between conception and functional sperm. Is this correct?

Atwood: As far as it goes. I meant to leave the impression that there is no particular reason to think that because we have differences in measured mutation rates among organisms, the rates of the elementary processes involved are correspondingly different. Instead, our estimates of the rates have different meanings; they are not really comparable without further assumptions. The question of relation to cell division is only one aspect of this. There are many others.

Neel: Cell division is the aspect that has come up repeatedly. What others?

Atwood: The question of direction of the mutation, whether it is forward or reverse. The size and environment of the mutable units. Selection effects at the cell level may play a role in some instances in metazoans, and then the various mistakes that can be made in measuring rates where difficult assumptions are required, as is often the case in man.

Neel: You are maintaining then that mutation, whatever it is, occurs at about the same rate in all manner of organisms?

Atwood: To put it more cautiously, we have no particular reason to think that it is not occurring at spontaneous rates having similar range.

Magni: On the other hand, the range of mutation frequency in the same organism is so wide that it is difficult to say whether the mutation rate is different from one organism to the other.

Benzer: Yes, that's right.

Neel: Per life cycle, or per cell division within the organism? I am not clear what our point of reference is in this attempt to say that all organisms have about the same mutation rate.

Atwood: I would say that even per cell division, the rates may well be comparable. One could find many examples of comparable rates if one looks for them.

Freese: I think you should stick to your cautious statement. The frequency of the elementary mutagenic processes may very well be different in different organisms. Responsible for this difference may be different base analogs or other chemicals. We have no reason to assume that the mutation rates should be alike.

Demerec: As you probably know, strains LT2 and LT7 of Salmonella differ considerably in their rates of spontaneous mutability. The reason for this difference is genetic, namely, the presence of at least one mutator factor and probably more than one in the LT7 strain. One of these factors has been identified as a gene located near threonine on the genetic map (22). Dr. C. Kirchner has observed that the supernatant of the strain carrying the mutator gene is mutagenic. He is working on extraction and analysis of the mutagenic substance. It appears to be a purine analogue.

Novick: One thing that disturbs me in this kind of discussion is that we look at a man with a much higher degree of care than we do a bacterium. If we looked at the bacterium with the same kind of care we look at a human, we probably would find all kinds of "mutations" occurring at 10^{-3} or thereabouts.

Goodgal: Yes, from the number of different suggestions which have been given, I just wonder if we can say anything with regard to the magnitude of the variables.

Zamenhof: The term, "mutation rate per generation," which should be universal if one wants to apply it also for evaluating the strength of mutagenic agents, cannot be applied when one deals with discontinuous events (43). Perhaps the mutation frequency, with the description of conditions, would be more universal as a term. But neither "the generation" nor "the time" is more important than any other of these conditions in many mutagenic processes. For the same mutagenic agent, you can have conditions where the term "per generation" is meaningful and other conditions where it is meaningless.

Auerbach: I just wanted to say that I agree, but it is only a terminological point. I always feel rather strongly about the word "mutation rate." When you have treated with X-rays or instantaneous or short exposure to mustard gas, this is meaningless. One should call it mutation frequency.

Atwood: Per dose?

Zamenhof: What I meant was this: there are mutational processes

which are single events, such as deamination of cytosine in viral RNA, and there are mutations which are composed of at least two steps, because the DNA cannot reproduce with the changes which one has inflicted by mutagenic agents, such as mustards. This means that the final fault does not occur at the moment of reaction with mustard, but this final fault, substitution of the wrong base, occurs later on at the moment of cell division, or DNA replication. Thus, there must be two steps for such a mutational process (43).

Auerbach: It may be so from what you call a mutation in the chemical sense, but from what I observe, I must draw the conclusion that mustard gas in spermatozoa, which presumably have a double helix of DNA, produces mutations, a high percentage of mutations, which affect the whole offspring. These cannot have occurred at the next division.

Atwood: Maybe the complementary strand was lethal.

Auerbach: Yes, this is a possibility. But there is, in fact, no evidence for believing that mustard gas-induced mutations must occur at cell division. There is a theoretical model under which this should occur, but if one talks of mutations, one should really talk of observable biological facts, and there are so far no facts that support this model.

Lederberg: You never observe a mutation, either.

Neel: Then we have arrived at the position that we don't really know what we're measuring when we measure mutation rates. We can't compare from one species to the next, but if we could compare, the denominator that would pull all rates closer together would be cell division.

Auerbach: There are mutations without cell division.

Zamenhof: That's what I just said.

Novick: The events which lead to mutations—

Auerbach: No, spontaneous mutations.

Magni: Why don't we separate the spontaneous and induced mutation rates? In the induced, we know what we are measuring, i.e., the effect of a given treatment on the frequency of mutants. For the spontaneous mutations we don't know exactly which is the frequency of the primary event.

Zamenhof: But spontaneous mutations are induced.

Magni: Well, that has to be proved.

Novick: That has been proved.

Atwood: I think that we ought to discuss actual evidence on this, of which there is some. Aaron, you might tell about the chloramphenicol-inhibited cells and how spontaneous mutation is occurring in them.

Novick: In the work you refer to, we studied only caffeine-induced mutations, not spontaneous (13). You will recall that in the presence of chloramphenicol the synthesis of protein ceases and that of nucleic acid continues. We showed that if caffeine is present with the chloramphenicol, mutations are induced, these being detected by removing the bacteria from chloramphenicol and plating them on appropriate selective agar. If the rate of mutation is expressed per unit of DNA formed, then the caffeine-induced rate is the same as in the absence of chloramphenicol. These experiments show that the synthesis of DNA is sufficient for mutations to occur and that the DNA formed in the presence of chloramphenicol is good DNA.

Auerbach: I would like to mention a very curious result that I got, using Kim Atwood's technique for scoring lethal mutations in Neurospora (2). I scored the spontaneous occurrence in desiccated spores. The mutation frequency increased absolutely linearly for seven months in these dried spores. It increased something like 1.3 per cent per week. Atwood's system is very ingenious. It is rather like the ClB method in Drosophila. You carry recessive lethals in a heterokaryotic condition and force them into the open by giving the right supplement for homokaryosis.

At the same time, I ran a series at 4° which was not very good, because here humidity was very poorly controlled. They did get humid, and the line was not so straight. It wobbled a bit. The astonishing thing was—but, unfortunately, I did it only once—at one point, say after four months or so, I took a sample of spores which had been sitting at 4°, put them at 30°, and left them there for, I think, a fortnight. Then I tested for mutation, and the frequency had gone right up to the level of the spores kept at high temperature. I want to repeat this experiment because it raises the possibility that in the cold some chemical mutagen is formed or some condition predisposing to mutation takes place, and that only the last step in mutagenesis has the observed high temperature coefficient.

Atwood: At the 4° temperature, something happens that then softens up whatever it is for mutation to occur later at 30°?

Auerbach: No, there are two points here. This is the second, and I'm not so sure about it because I did it only once, but it was very striking. This curve shows that mutation occurs not only without cell division but also without chromosome replication, because Atwood's system is such that if, say, this is the double helix, and suppose the mutation occurred here, it would give rise to a colony which is half-mutated. Now, this is the only good system that I know of in which

this possibility is excluded, because a mosaic would not be scored as a recessive lethal.

Lederberg: You are also saying that it is not a duplex model, then. You have to make more drastic revisions than this.

Auerbach: That's the difficulty. But I'm not so sure with these lethals whether there can be some effect on both strands.

Novick: It's too bad that these experiments can't be done with phage because it would be nice to know how wide these mutants are, what is the nature of such mutations.

Lederberg: Experiments indicating one strand mutagenic effects have been done with phage (30).

Novick: I meant experiments of the kind where—

Atwood: —where you let them sit around and mutate.

Freese: I would say one has two extreme possibilities. If one uses a base analog as mutagen, it seems rather clear that the increase of the frequency is proportional to the *number of cell divisions*. In contrast, if one uses nitrous acid, the frequency of mutants is proportional to the *time of treatment*, provided that one lets the cells go through one or two more generations that express the change.

The spontaneous effects may be intermediate; we may have two spontaneous mutagenic effects, one which is proportional to the division time, and another which is proportional to the time the cell stands around. How can we express this quantitatively? We probably have to use two dimensions for the abscissas, one dimension being the division time, and the other dimension being the time of exposure of the cell.

Zamenhof: Suppose you don't have the cell, but you have mutation of DNA *in vitro* induced by nitrous acid; would you call it mutation without cell division, if there is no cell?

Freese: Then you talk about the DNA division.

Auerbach: Did you ever get mutations in resting bacteria? You found the mutation frequency was proportional to time, but did you go down to complete resting bacteria?

Novick: What we found was, that as we reduced the growth rate and increased the generation time, the mutation rate remained constant per hour until a generation time of about 15 hours. Further increase in the generation time was accompanied by a decrease, a proportional decrease, in the mutation rate per hour. I can understand this fall at very long generation times by imagining that the bacteria cannot regulate themselves to grow at an arbitrarily slow rate, and when the chemostat is operated at generation times longer than 15

hours, the concentration of the limiting substrate gets so low that the bacteria give up the ghost and stop growing. The concentration of limiting growth factor then builds up to a high enough level to start the bacteria growing again. Thus the bacteria grow only part of the time. For example, at an apparent 30-hour generation time, the bacteria are growing only half the time, so the mutation rate per hour is one half.

Auerbach: Well, wouldn't Ryan's experiments agree with what Dr. Freese said?—because he got discontinuous mutation frequencies, I think it was a factor of 40 or so, between mutations occurring in the stationary phase of bacteria and mutations occurring in the "log" phase (31).

Neel: Could somebody describe Ryan's experiment for us who don't know it?

Auerbach: As far as I remember, he did this work exclusively with histidine reversions. He compared spontaneous mutation frequencies in growing and resting bacteria, and he checked on residual growth in various ways, for instance by the use of chloramphenicol. There were mutations in resting bacteria, but as I said they were very much less frequent than during the log phase. He used penicillin to check on growth, as to whether or not there was growth, and he did get fewer mutations, but he got an occurrence of mutations in that stage.

Freese: But he also had shown that the DNA duplicated.

Auerbach: He just showed that there was an increase in DNA which might be DNA precursors. I don't think he showed that the chromosome duplicated.

Goldstein: May I add an experiment to this which bears on the same question? In our studies on amino acid starvation in *E. coli* auxotrophs, one can abruptly stop protein and RNA synthesis by the withdrawal of the required amino acid and maintain this condition of starvation for a period of one and a half to three hours. Three hours in this organism corresponds to approximately four to five generations time.

In the first half hour of this amino acid deprivation, there is about a 30 per cent increase in the DNA, but then it remains stable at this level for the rest of the starvation period. These cells are all physiologically competent, because if they are refed with the missing amino acid at the end of three hours, then protein, RNA, and DNA synthesis immediately resume without lag, and at the original rate.

We did do a study in this system on the frequency of streptomycin resistance during the period of starvation, in which we could easily have picked up the anticipated increase in the number of mutants if

it had been a time-dependent phenomenon, but, in fact, when one made allowance for the 30 per cent increase in DNA in the very early part of the starvation period, then there was no further increase in the number of mutants, which would suggest, again, in this particular system, continued DNA replication was essential for the continued production of mutations.

Zamenhof: Perhaps we could agree on what is actually called mutation. Suppose there is a mutagenic process, and then the cells are stored without cell division. Finally the cells divide, and one detects mutation; which of these steps, or all three, should be called mutation? In the first process, some change is registered in DNA. To give an example we heated the cells to 155° which must have registered some change in DNA (43), then we stored the cells for a month, and finally we let them divide, whereupon the mutation was discovered. Now, which of these processes should be called a mutational process?

Auerbach: The first.

Benzer: Why? As long as you are unable to separate them, how can you assign a developmental role to one or the other?

Auerbach: If you can correlate the frequency of the final event with the type of treatment you use, then the primary step must be due to this treatment, and one can call that mutation.

Zamenhof: Because the second step might be quite trivial.

Auerbach: What I call the primary step in mutation must be due to the treatment.

Benzer: But this distinction could come only as a result of investigation. The mere observation does not enable you to project into it your own ideas as to the stage of the primary event.

Auerbach: No, whether the actual change in base sequence is induced at this time or not, we cannot say, but whatever the chain of events is afterwards, if the same final event is always correlated with the same treatment, then the essential process must have occurred during treatment.

Goodgal: No, that doesn't follow, because if there are a number of different steps in the process of mutation, then you may be able to induce mutation at every one of them. You can't say anything until you define the steps.

Zamenhof: But the second may be trivial. Cell division may always be the same, and in that case it is not the rate-determining step, as Dr. Atwood said. The rate-determining step would be actually the first treatment with mutagen. This step decides what the final outcome will be.

Novick: May I ask a question? Let's imagine, as a result of heating your bacteria, you produce bacteria that have become metabolically abnormal for a while after growth begins. The longer you heat the bacteria, the higher the temperature, the larger the fraction of the population that becomes metabolically abnormal. Let's say that as a result of being metabolically abnormal, mutation is more probable. The mutation actually occurs during growth, in the relatively small fraction of the population which is metabolically abnormal. Now, the longer you heat, the more mutations you apparently will produce, as you said.

Now, when did the mutation occur? When you heated it or when you grew it?

Zamenhof: Suppose I extract DNA after heating, without letting the dry cells divide, and prove that the change is already there. If upon the introduction of this DNA to normal bacteria, I could transform them to a mutant?

Atwood: Then we would have a fact.

Auerbach: I quite agree. Where you can analyze the process in such a way that we find out which part of the whole process produces this change in base sequence, then we can say where the mutation occurs. But we usually can't at this stage. It's a question of terminology at the moment.

Benzer: There seems to be some disagreement as to what the title of the symposium refers to, namely the word, "mutation." Could the Chairman tell us what it is?

Atwood: May I answer that?

Neel: Dr. Atwood is undertaking to answer your question.

Atwood: I suggested in a private discussion a few minutes ago that one ought to use only operational terms, but since some operational terms for mutation are going to be different, depending on the user, we would like to know what are your operational terms? What does a mutation mean in your system?

Benzer: To me, something to be called a mutation has to involve a change and this change has to be demonstrated to be heritable.

Atwood: And that is all?

Benzer: There are all kinds of subclassifications after that, but, to me, these two criteria are necessary. Without them one cannot use the word.

Neel: You said it has to involve a change. A change in what?

Benzer: A change in characteristics.

Stern: Would you call the origin of petites in yeast a mutation?

This seems to be caused by loss of some cytoplasmic material. It is a change, it is inheritable, but it is in a completely different class.

Benzer: I would be inclined to class that as a mutation of that particular type.

Zamenhof: Another thing—you have to remove the mutagen, for if the cell changes only while the mutagens are around, it may be just a phenotype change. If you put 5-bromouracil in a medium, it produces some change in all cells, but it is not a mutation because the cells return to normal upon removal of 5-bromouracil (45).

Benzer: Of course. That is implied in the statement of its being heritable.

Novick: These are sufficient conditions, but it is not necessary that—

Benzer: I insist that they are necessary. Whether they should be taken as sufficient is another matter. Personally, I would be inclined to do so and let the precise subcategory await further investigation in each case.

Lederberg: Why do you object to this being included for the case of differentiation? I find that perfectly congenial.

Auerbach: But if one has an operational method of classifying things, why should one not use it? We have a method for determining whether it is a change in the DNA information or whether it is not, by making the additional postulate that it should either segregate or recombine.

Lederberg: You can have nucleic mutations and nonnucleic mutations on this formulation.

Auerbach: As they are distinguishable operationally, do you not want to distinguish between them?

Lederberg: Yes, it is a classification.

Neel: Are we agreed, then, that from here on a mutation is defined as an event manifested by a heritable change in the organism?

Auerbach: I should like to include in the definition that it shows that it is affecting the nuclear information by either segregating according to Mendel's law or by recombining.

Stern: These definitions include chromosome aberrations. Mongolism is a change, it is inheritable, and we now understand its genetic nature. It is caused by an extra chromosome. Its frequency may be positively correlated with the number of cell divisions since it originates during cell divisions. To include its origin in one definition of mutation would lump together the kind of changes which affect the molecular configuration of DNA and those which are simply gross changes in the quantity of DNA material. Since we are aware of

different types of changes which fit your definition, Dr. Benzer, we should separate them from one another.

Freese: I think we cannot really split them. In bacteria, for example, we really don't know in many cases what kind of mutations we have, according to your definition.

Stern: In certain cases you can and in others, you can't.

Freese: Yes, so the word, "mutation," is then a general one, and we can use, in addition to this, a further subclassification.

Auerbach: But I think in many bacteria one can determine by recombination or transformation or transduction whether a mutation is chromosomal or genic.

Freese: But there are some bacteria which cannot be transduced or which you cannot cross as yet, and, actually, this is the majority of them.

Lederberg: I think we've reached the status of information about the importance of nucleic acid that, while all of us want to have lurking in the backs of our minds some reservations about the extent of the validity of our proofs, it's just nonsense for us to waste our time in casuistic arguments about whether we have proven that this, that, and every other case of a genetic change has involved nucleic information. Why don't we get down to brass tacks? Most of what we're going to talk about in the next couple of days is going to be changes in nucleic information. There are a few areas where we don't know this for sure, and we can question that point.

Demerec: If you feel that discussion about terminology or definition has been carried far enough, I would like to ask a very specific question, which will probably be easier to answer than to define a term like "gene." That question is this: Is there any evidence of the existence or nonexistence of specificity in forward mutations?

Auerbach: The phage work.

Demerec: Oh, no, those are all in reverse!

Auerbach: No, you have them in both directions; the *r II*.

Benzer: There is fantastic specificity in both directions.

Demerec: What is the evidence?

Benzer: In *r II*, the A cistron has twice the mutability of the B cistron.

Demerec: Twice as frequent?

Benzer: Yes.

Auerbach: But is it twice as long, then?

Benzer: It is also twice as long, approximately.

Auerbach: But that is not specificity.

Benzer: On what level do you want the specificity? Dr. Demerec asked for specificity at the level of the locus.

Auerbach: I was looking at this question from the point of view of my discussion tomorrow, and I found only one piece of evidence of mutagen specificity in the sense which Dr. Demerec, I am sure, means: that when you compare several mutagens, you get a pattern which differs between loci in forward mutations. This has been found by Heslot (18). He works with fission yeast, *Saccharomyces pombe*, and he scored for forward mutations at four different adenine loci. The method of detection is the same that Hershel Roman first used in yeast. He starts with a strain that carries a purple adenine mutation so that the colonies look purple. If the chain of reactions leading to adenine is interrupted at some earlier stage by mutation at one of the other loci, then the purple pigment is not formed, and the colonies are white. It is a very neat method. He used four mutagens; UV, ethyl sulfate, isopropyl methane sulfonate, and ethylene imine. I shall give here his main results. I shall leave the ad-5 locus away because it mutated rarely with all four mutagens. In parentheses after the actual numbers of mutants obtained I shall insert the relative frequencies of mutations at the ad-1 and ad-3 loci compared with those at the ad-4 locus; this makes a comparison easier.

	ad-1	ad-3	ad-4
UV	19(1)	20(1)	19(1)
Ethyl sulfate	56(1.5)	84(2)	40(1)
Isopropyl methane sulfonate	57(3.5)	41(2.5)	16(1)
Ethylene imine	18(2)	21(2)	10(1)

When statistical tests were made they showed that the distribution of mutations after treatment with ethylene imine was significantly different from the distribution after treatment with UV or ethyl sulfate.

Cotterman: These are frequencies of mutant cells per how many? What is the total cell count?

Auerbach: I don't remember. I don't think he even said, but he must have taken that into account when calculating the statistical significance.

Freese: Actually this is not so surprising, because we have the tremendous differences in mutation frequencies if we look at one particular site. If you assume, let's say, 500 or 1000 sites in a gene, or

in a cistron, then you might very well end up with differences of that order.

Auerbach: Oh, yes, certainly.

Goldstein: I might add that we have evidence in the case of the mutator strain of *E. coli* that we have been working with, in which one is studying spontaneously high mutability in one and the same organism, that there is a variation of several orders of magnitude in the mutability of different sites for forward mutation. To give you a few examples, the incidence of lac^+ to lac^- is of the order of 10^{-4}. The incidence of T4 resistance is about 4×10^{-3}. The incidence of resistance to a variety of other antibiotics, where we don't know the specific genes involved, for example, penicillin, erythromycin, chloramphenicol, is very low, about 10^{-7}. The incidence of total auxotrophs is about 3×10^{-3}, of which the largest are histidine auxotrophs, and if one took the forward rate on any particular one, such as uracil, for example, this would be around 10^{-4} or 10^{-5}.

Auerbach: The first ones you mentioned, are they known to be due to mutations at a particular locus, like reversions? Or could one of them be due to mutations at several loci and the other one only to mutations at one locus?

Goldstein: None of these has been subjected to genetic test. The lac^- changes include both galactosidase and permease.

Neel: Perhaps I can try now to answer Dr. Benzer's question as to whether today we have been discussing what it was intended we should discuss under the heading of "mutation." It was hoped that today would bring out precisely what it has brought out; namely, that persons who work with different organisms think along somewhat different lines when they speak of a mutation. I think that the ultimate in how far your thinking can go has come out in the last ten minutes, where, in discussing differences in locus rates, we have wondered whether the number of nucleotides comprising the loci in question are the same.

There is one aspect of this problem of measuring mutation, and particularly in comparative mutation rates for man to mouse or Drosophila to bacteria, that has not been touched on. This is the question of whether, from one species to the next, we are detecting with our usual laboratory techniques the same proportion of all the mutations which are occurring.

Let me state an extreme case. Possibly the structure of a bacterium is such that any change in the genetic information results in a phenotypic effect that can be picked up by a suitable technical method,

whereas in man there are, perhaps, a sufficient number of homeostatic mechanisms that we can readily detect only a small minority of the changes in the genetic information.

Demerec: A good answer to your question is to be found in an observation made in pre-X-ray days by Timofeff-Ressovsky: that in *Drosophila funebris* visible mutants are extremely rare.

Neel: Dr. Demerec, I can't resist saying that the first paper which I ever wrote was on the mutants of *D. funebris* (24). It does indeed seem to be the case, that despite a considerable amount of work, *D. funebris* has yielded only a fraction of the clearcut, nice, clean traits seen in *D. melanogaster*. Almost all the mutants recognized have been extremely susceptible in their manifestations to temperature and nutrition. What this means in terms of the structure of the genetic material, who can say?

Glass: Was there a comparison in respect to lethal rates, too?

Neel: No.

Auerbach: Wouldn't the difference be rather in the biochemistry of the species than in the genetic material?

Stern: Some of the types looked for were body or eye color mutants which are frequent in melanogaster but were not found in funebris.

Auerbach: But this may be due to a difference in development between the species. I remember a striking case of this kind in Chlamydomonas. Although mutations to amino acid requirements were found in Reinhardi, Gowans (15) was not able to induce them in eugametos. Yet he obtained other types of requirement as mutations. It seemed to me that this difference might have something to do with the metabolism of these two species, perhaps something which makes this kind of mutation lethal to eugametos.

Lederberg: Cryptic autopolypoidy would be much more like it.

Auerbach: But he got other mutations.

Lederberg: But not the entire gene; part of it.

Auerbach: But all the amino acid determinants would have to be in one particular part of it.

Lederberg: We are beginning to believe that now.

Neel: But when you say it may be due to a difference in metabolism, ultimately, I take it, this goes back to the genetic material. If there are species which have a good deal of cryptic autopolypoidy, this certainly does interfere with comparing mutation rates from one to the next, doesn't it?

Auerbach: But that wouldn't happen in Drosophila, by segregation ratios.

Neel: Yet a considerable number of little repeat regions are known in *D. melanogaster.*

Auerbach: Yes, that is true.

Lederberg: Jim, did I understand correctly that you were suggesting that the greatest number of mutations would reach their expression in man and not, perhaps, in bacteria? You were implying that lethals would not be the most common event of the genetic imbalance in man?

Neel: I was not implying anything so much as raising a question, whether the nature of Drosophila or some bacteria may be such that any nucleotide change will result in a detectable effect whereas, in another species, a nucleotide change will not have the same probability of a phenotypic effect.

Magni: It depends also to a great extent on the technique for scoring mutants. For a bacterium unable to grow on a minimal medium, for example, the number of mutants one could be able to pick up should be much lower than for *E. coli,* and this would not be due to a peculiarity of the organism but rather to our technical approach to the problem.

Glass: To answer Joshua Lederberg's question in another way, we most certainly do not find lethals as the most common kind of mutation in Drosophila. In those studies that have been carried out to get comparable figures, detrimentals that do not produce lethal effects are more common, much more common, than lethals (23, 41). It is largely because of the nature of the technique of screening in microbial genetics that you get lethals as the most common type of mutation. Isn't that so?

Lederberg: It's an interesting point, I think, that has been suggested before—that the thousandfold difference in the information content of bacteria and of man does not represent a whit of difference in biochemical enzymatic information, but just in the more subtle controls, and these may perhaps often be partial systematic effects.

Goodgal: I don't think the definition of lethals in the microorganism is quite valid, because I think a number of people are finding now that you can separate mutation from lethal by controlling things like RNA synthesis, so you get a very high frequency of mutation with very little killing.

Lederberg: The word "lethals" has been used here to mean lethal mutation, and it should be distinguished from bactericidal effect.

Goodgal: Yes, but if you're getting mutations without very much killing, then you can't be getting lethal mutations.

Atwood: If you have a treatment that gives no apparent killing and gives a high rate of total auxotrophy, let's say 10^{-4}, well, if the actual number of auxotrophs formed carries with it a thousand times that many lethals, you wouldn't detect it, because you would still have less than 1 per cent of the bacteria killed, and nobody detects that difference by plating. You would still say there is no killing with this mutagen.

Goodgal: Yes, essentially, that is correct.

Atwood: So that you can't tell an irreparable recessive lethal except by a special method such as trying to stabilize the diploid.

Neel: Those of us who work with man are intrigued by this discussion of how difficult it is to define a lethal in bacteria. At the risk of being overly sweeping, I have the feeling that the range of mutations with which we can work in man is so restricted in one way or another, and that the range with which you work in bacteria is, perhaps, so restricted in its way, that any attempt to compare mutation rates in two species is premature at the present time.

Auerbach: But one doesn't compare the over-all rates. One usually compares mutation frequencies at individual loci, or what one thinks are individual loci.

Neel: My remarks apply to individual loci, as best we can come to grips with them in man.

Auerbach: Yes, but I don't really see the difference; once you can recognize a mutation at an individual locus, it doesn't matter whether it is an eye color in Drosophila or hemophilia in man.

Neel: You mentioned hemophilia in man. It is a fact that what was hemophilia ten years ago is now one-third another disease that we call Christmas disease. In man we never know when we are working with a single locus, and we never know how much of the mutation spectrum at that locus we are picking up.

Auerbach: I think that many of these difficulties disappear when one considers individual loci rather than the whole genome. One cannot compare all mutations in man with all mutations in Drosophila; this is meaningless.

Neel: I wish I felt as confident as Dr. Auerbach, that these difficulties disappear when we talk of the individual locus.

Auerbach: Some of them disappear. I think Kim has enumerated those which remain, but I think the additional ones which you made will largely disappear if one restricts oneself to a comparison of mutation frequencies at what one thinks are known loci. I know that the estimate of mutation frequency to hemophilia has changed, but only by a factor of 2 or 3.

Motulsky: With man, you can almost generalize that every genetic disease that has been investigated biochemically during the last few years has turned out to be heterogeneous.

Auerbach: But what is the factor? It won't be more than 2 or 3.

Neel: Professor Dobzhansky, will you summarize now?

Summary of Discussion

Dobzhansky: Last night, when our Chairman asked me to undertake the function of summarizing, I reluctantly agreed. I certainly did not know what I was getting into. I do not undertake the impossible, but shall try to comment on some of the concluding sentences of Atwood's presentation. The way we look at mutations, the way we go about studying them, depends on the purposes of the study. I think that the discussion this afternoon was revolving around this point.

In dealing with a difficult material like the human species, and being interested in the role of mutation in the maintenance of the genetic load, we adopt a different definition of mutation and go differently about studying mutations than when we want to find the causes of mutation.

It would be rather unfortunate to go back to the state of things in 1905 and define mutation as any and all heritable changes. As far as I can see, this is, however, the definition which we have to adopt for quite a number of purposes, especially in dealing with man. Obviously, we must try to analyze what sort of heritable changes we are observing in order to distinguish the chromosomal changes, the cytoplasmic changes, and the changes which, for want of a better explanation, we ascribe to qualitative alterations of the gene structure.

I think that our Chairman tried, unsuccessfully, in the last fifteen minutes to start a discussion of the differences between major mutations which produce clearly visible, clearly classifiable phenotypic effects, and the so-called polygenic mutations. About the latter we know very little, and, in many organisms, nothing at all.

An analogy has been suggested between the study of mutation and the study of such an obvious phenomenon as death. A statistician or a statistical epidemiologist will try to ascertain how many people died, of let us say tuberculosis, during a given time interval in a given territory, to discover whether the mortality is periodic or shows no regular periodicity, whether the increases and the decreases in the mortality can be correlated with some climatic, economic, sociological, and other factors, etc. But sooner or later we shall want to know what

causes the disease called tuberculosis, under what condition it does and does not lead to death, what are the changes which it causes in organism, etc. The problems faced with mutations are fairly similar. The first, and perhaps the simplest, question that is asked is how frequent mutations are. Then we shall inquire at what stages of the life cycle do mutations occur. Let me remind you of some of the figures given by Dr. Atwood. Mutation frequencies per gene per generation in man are usually of the order 10^{-4} or 10^{-5}. In microorganisms the orders of frequency seem to be 10^{-8} or 10^{-9}. Little information concerning the frequencies of mutations exist for organisms intermediate between man and bacteria. Being an old-fashioned naturalist, I am tempted to remind this group that such organisms are quite numerous. A fair amount of data exist only in Drosophila. In about half a dozen species of this genus of flies, the mutation rates for changes producing lethal effects are pretty uniform. They are of the order of 10^{-5} per gene per generation, very much like human mutations. Mutation frequencies for other types of changes are little known. Really next to nothing is known for mutations of the polygenic type, even in Drosophila. It is, in my view, a remarkable fact that the mutation frequencies seem to be so similar in organisms as different as man and Drosophila.

Now, where does mutation occur? This is a problem which is important for purposes of analyzing the cause of mutation. Whether we measure the frequency of mutation per generation or per unit time will depend on what we wish to do with our data. I am not sure that I understood correctly the drift of Dr. Atwood's presentation. I take it that his argument tends, on the whole, to the conclusion that mutation occurs at the time when cells divide. We then have to enter into the analysis of the problem whether mutations accumulate with age and, if so, what are the mechanisms of the age accumulation. Here it becomes important to know how frequently mutations arise in males and in females, whether they arise at meiosis or at mitosis, during the period of rapid growth of the organism, after the rapid growth has been completed, in the spermatozoa which are being produced in the testis, in the spermatozoa during the storage period, as in Drosophila, etc.

The most interesting and suggestive data which Dr. Atwood has presented with respect to the presumed mutation rate in the erythrocytes seem to me very promising as a possible technique of going at this problem in the future, and I take it his estimate of the mutation rate obtained from the progression curve, 10^{-8}, 10^{-9}, per cell per hour, has to be taken as the first and a very rough approximation.

I may, perhaps, re-emphasize one of the points made by our Chairman. One of the unsolved problems is whether the methods of detection of mutations in different organisms are really capable of producing estimates that are reasonably comparable. This is quite important in some studies. I have particularly in mind studies on the genetic loads in different populations. This is one of the key problems which genetics faces. Do different organisms produce mutations of comparable kinds? If so, how do the frequencies of these different kinds of mutations compare in different organisms? About these matters we know at present next to nothing at all.

Neel: Thank you, Professor Dobzhansky. You have done what we hoped you would do, with remarkable clarity and brevity. Anything else would be anticlimactic.

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MUTAGENESIS, WITH PARTICULAR REFERENCE TO CHEMICAL FACTORS

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I want to say a few words about the summary which I sent out, because you may have thought it a bit carping or too negative. One reason I wrote it like that was that I was asked to be challenging, so I thought I would make it challenging. But there are two other reasons. One is that, having now really gone into mutation work with microorganisms, I am puzzled by quite a lot of difficulties which do not seem to have found much attention in the past, and I hope we will get them discussed here. Secondly, I believe that the work on phage transforming principle, bacteriophage, and tobacco mosaic virus has been so beautiful and so fascinating in the light it throws on the nature of the genetic material that there is a danger that the biologists will turn over their results to people with chemical training and forget that the genetic material, after all, sits in the cell and that lots of things happen in the cell which would not happen to anything *in vitro*. It is implicit in much work—the assumption which, to me, sounds naive from a biological point of view, that if you put a chemical into a cell, it makes straight for the DNA and reacts with it, and whatever else happens is a rather negligible and deplorable accident, which one should put out of the way.

I have arranged what I wanted to say under a number of headings. I can already see from the previous volume that I won't have very much time to speak uninterruptedly, but I welcome this very much. However, I thought I would give myself a chance of talking about this for about five minutes, by doing something very uncontroversial; that is, by quickly running over the history of chemical mutagenesis.

We can ask ourselves one question here; that is, how important is a good theory of the genetic material for finding a good mutagen? The first principle, and one which is still used very much for the testing

of substances for mutagenic action, is pharmacological. Mustard gas was discovered because, pharmacologically, it resembles X-rays in the kind of burns it produces. Then followed a whole list of similar substances. Simply on pharmacological principles, other substances were found to be mutagenic; for instance, allyl *iso*thiocyanate was tested because it was vesicant. In 1947, I got a letter from a German Ph.D. student who sent me a sealed little vial with a chemical in it, and thought it should be tried because it was vesicant. I had so much else to do that I forgot all about it. Then, about ten years later, ethylene imine turned out to be a good mutagen and this somehow seemed to ring a bell, so I hunted through my chemical cupboard, and there was this little vial. It was ethylene imine which had already been suggested on purely pharmacological grounds.

Pharmacological research is still the source of an ever-increasing number of the so-called alkylating agents that produce mutations. All of them are also carcinostatic, but this correlation is one-sided and somewhat biased. What happens usually is that when a substance is found to be carcinostatic, it is turned over to a geneticist, mainly the Fahmys (21) at the Chester-Beatty Institute in London, and then it is tested for mutagenicity. Usually it is found to be mutagenic. I think the connecting link between the two properties is the ability to break chromosomes.

Also on pharmacological grounds, a number of alkaloids have been tested successfully for mutagenic ability. Oehlkers (57) in Germany produced chromosome rearrangements with scopolamine, morphine, and similar substances. Clark (11) in Australia detected another group of mutagenic alkaloids on pharmacological grounds, this time the suggestion came from veterinary medicine. Certain pyrrolizidine alkaloids, for instance heliotrin, are of concern to sheep breeders because they cause liver disease in sheep. He tested some of them on Drosophila and obtained very high mutation frequencies indeed. I want to give this simply as a suggestion that there may be whole groups of substances that have never been tapped for mutagens.

After this, I come to substances which were discovered because of theories as to how a mutagen should act. One of the theories which was developed in connection with the alkylating agents was the idea that cross-linkage is necessary for mutation. This idea came from wool research, where it had been found that some of these substances cross-link protein fibers. Then the cancer workers found a very impressive correlation between the number of functional groups in these compounds and their carcinostatic ability. In fact, Professor Haddow

told me recently that there has been hardly any exception to the rule that only two- or three-armed compounds are carcinostatic.

This was taken over into the theory of mutagenesis, but there it proved to be wrong. A one-armed mustard or other one-armed alkylating agent may be an excellent mutagen, even better than the corresponding two-armed compound. But the point is that some very good mutagens were discovered on the basis of the cross-linking theory, and only later were the corresponding one-armed compounds tested and, sometimes, found to be also very effective as mutagens. Ethylene oxide was tested after diepoxybutane had proved to be mutagenic. TEM, triethylene imine, was tested before ethylene imine. At least, this is so for countries west of the Iron Curtain.

In Russia the story of chemical mutagenesis was different, and this brings me to the third principle on which mutagens were detected. The first was pharmacological, the second was the cross-linkage theory. The third forms the basis of Rapoport's (59) work in Russia, which became known in the west only years after it had been carried out and when Rapoport himself was no longer allowed to work along these lines. Rapoport, like everybody else at that time, believed that the specificity of the genetic material resides in the protein. So he searched for chemicals that would act on proteins, and one of the many that he tested with success on Drosophila was ethylene imine.

On the basis of the same theory, Rapoport (58) discovered the mutagenic action of formaldehyde. It is interesting to remember that, likewise on the basis of this theory, tests for mutagenic ability of nitrites were carried out long before the spectacular success with this substance in recent years. In 1939, Thom and Steinberg (75) produced mutations in Aspergillus with nitrites. When formaldehyde had been found to be mutagenic, Haldane suggested trying nitrites because these, like formaldehyde, react with the amino groups of proteins. Robertson and Rendel in our Institute tried nitrites on Drosophila. The first series was very promising, but later series were negative, and they abandoned the work.

We come now to theories that are closer to what we now think is the truth about the nature of the genetic material. Even before Avery's work so strongly implicated nucleic acid, it was of course known that nucleic acid is an important part of the genetic material. This started off experiments with purines or pyrimidines or with substances known to react with nucleic acid, like pyronine and acriflavine. Many of them were found to be chromosome breakers and mutagens.

There is one claim which has been going on for, I think, twenty

years and has not been substantiated as yet in my opinion. This is Gershenson's claim that he had produced mutations in Drosophila by adding thymonucleic acid to the medium (27). Rapoport tried to repeat this, and somebody in Muller's laboratory tried to repeat it, but without success. Now, however, when genetics has again started in Russia, Gershenson has made the same claim on the basis of data which I did not find very convincing. However, he wrote to me about it and sent me a sample of his calf nucleic acid, and assured me that if we tested it the way he did, it would prove mutagenic. I think somebody in our laboratory will try it.

After this, then, come the mutagens which were tested on the basis of our knowledge, or what we believe is our knowledge now, that DNA is the important part of the genetic material. The other day I was looking through some old notes I made on this point. I was amused to see that immediately after Avery had published his first data on transformation—I believe it was in 1944—Muller wrote to me suggesting that somebody should test deoxyribonuclease for mutagenic ability. I was busy with other chemicals then, and this suggestion was not followed up at the time. Last year Kaufman (39) had the same idea, and DNAase did, indeed, turn out to be mutagenic for Drosophila.

Then, finally, nitrous acid has come back again, and this time on the basis of what we think is a correct theory of how it acts on DNA and RNA (28). It has been used with very spectacular success.

There is one conclusion which I think one should draw from all this; in the history of chemical mutagenesis, the wrong theories have very often led to the selection of very good mutagens. I think this should warn us to be cautious, for it means that if we have a theory about mutagenesis and, on the basis of this theory, choose a mutagen, and it acts, this does not prove the theory. It doesn't disprove it, but it doesn't go very far toward proving it. Ethylene imine was used with success on the basis of at least three different theories.

Perhaps there is a practical question here, but I don't know whether you want to discuss it; that is, what mutagens one should choose for future work. Apparently there is a vast array already to choose from, and I am convinced that there are whole additional groups of substances that contain mutagens. I think, from a practical point of view, one should look for mutagens among substances which are likely either to cause damage by producing mutations or to be helpful in inducing mutations where they can be useful; for instance, in microorganisms.

However, from a theoretical point of view, I think the choice of mutagen depends on the attitude one has to the study of mutations. It

is obvious that if one is interested in using mutagens as a decoding tool, one should limit oneself to those which are most likely to act directly on the nucleotides, and this is the thing I want to discuss next. But, as I said, I have a more biological approach to it, and I personally am very interested in the many steps which go on in a cell when a mutation occurs.

What I wish to discuss now is how do these mutagens act? I think that nitrous acid is one substance for which even I must assume that, at least in the tobacco mosaic virus, in the polio virus, and in the transforming principle, it acts in the way in which it was predicted it would act, namely, by deamination of the nucleic acid bases. Vielmetter and Schuster (76) in Tübingen think they can determine which base transformations are the actual mutagenic ones. The principle they use is this. They establish a correlation between the effect of pH on deamination of the bases *in vitro* and mutation or killing of T2, and they come to the conclusion that deamination of adenine and hydroxymethyl cytosine acts mutagenically and deamination of guanine is lethal. I cannot judge this work because of my lack of chemical knowledge. I wonder whether Dr. Freese knows it.

Freese: Yes, I do.

Auerbach: What do you feel about it?

Freese: It shows a correlation between the pH dependence of the mutagenic effect and the pH dependence of the deamination of the three bases, and that correlation is very good.

Auerbach: It seems to be all right, also, because the phage work really suggests this action of nitrous acid is directly on the DNA. I think that is very nicely suggested by comparison of the action of nitrous acid on the two-stranded DNA of T4 (77) and the one-stranded one of Tessman's (74) phage.

Freese: That is $\phi \times 174$. There is only one DNA strand in this phage.

Auerbach: Well, anyway, when the one-stranded phage was treated with nitrous acid, there were only complete mutants, but when the two-stranded ones were treated, there was a high proportion of mosaics, as one would expect. In fact, the proportion in Vielmetter's work was rather low, something like 50 per cent mottled plaques and 50 per cent complete plaques, whereas one would expect, of course, a much higher percentage of mottled ones. But I think that Vielmetter has a plausible explanation in assuming that, in many cases where he gets a complete plaque, the one strand of the DNA has been knocked out by a lethal hit, so the mutant is produced only from the other one.

Freese: Perhaps, I should say something here about some experi-

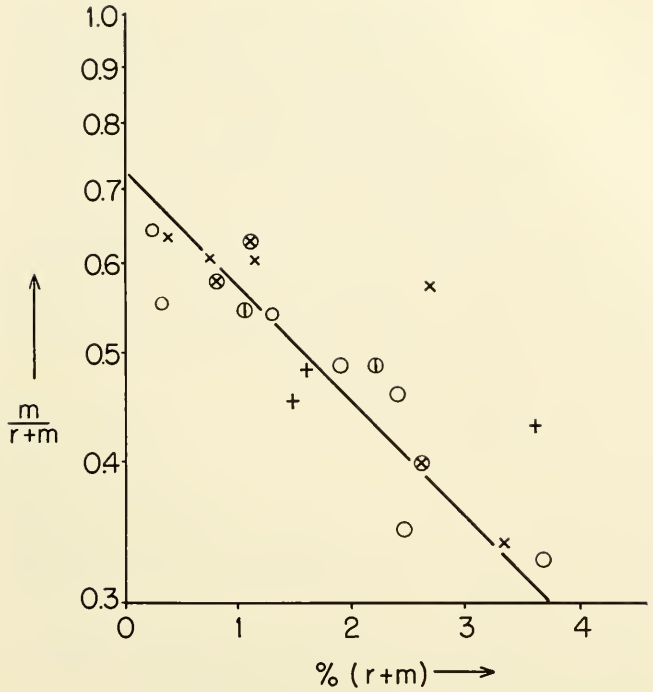


Figure 15. Decrease in the fraction of mottled plaques with increasing mutational hits. Points indicated by different symbols belong to independent experiments.
m = frequency of mottled plaques per plaque forming phage.
r = frequency of r plaques per plaque forming phage.

ments that we have done with hydroxylamine (26) which are concerned with such mosaics. Hydroxylamine is a mutagen which acts preferentially on cytosine bases. We have used it on phage T4 similar to the work of Tessman and Vielmetter and Schuster with nitrous acids. But we have also determined the ratio of mottled plaques versus the sum of *r* and mottled plaques with respect to the frequency of the sums of *r* and mottled plaques. *r* plaques represent the pure mutants while the mottled plaques correspond to your mosaics (Fig. 15). I don't want to go into the reason why I have chosen this particular abscissa; it has to do with other lethal properties of hydroxylamine. We find a lot of scattered points but we can put approximately a straight line through them.

When we extrapolate this straight line to the origin we do not get the value 1, as we would expect if all the mutants induced are mosaics, but we get about .7 to .8. This probably means that even without mutagenic treatment a certain percentage of one of the two DNA strands are thrown away in the bacterium. That is at least one explanation of this observation.

Lederberg: Couldn't you already have 30 per cent of spontaneous lesions in one strand? In other words, you are extrapolating back to zero external dose or intrinsic dose of similar lesions already present. This is not different from your statement, but it has the somewhat different connotation that this per cent of losses could be an accident and not a determinant.

Freese: That's right, it could be.

Goodgal: You would conclude from this that both strands of the DNA are active?

Freese: Well, I think you have to be very careful with the word "active." The observation of mottled plaques indicates that both strands of the DNA give rise to progeny phages. It does not follow that both strands are functionally active themselves.

Goodgal: No, but genetically.

Freese: Yes, genetically. They can give rise to mottled plaques.

Auerbach: Can one see that they are half and half?

Freese: You can isolate the phages from the mottled plaques and replate them; then you get varying mixtures of *r* and wild-type phages. It is not half and half. The ratio varies.

Auerbach: That may be just genetic drift or selection.

Freese: Well, there are at least two factors. You have selection in a plaque and selection in a bacterium. Some of the phage genomes may duplicate faster or more often than others. Some may mature earlier than others. So you have some variance there.

Novick: There was some confusion in the discussion of this figure, or it confused me. The origin is infinite dose, isn't that correct?—that is, dose decreasing as it goes on.

Freese: No, the origin is zero dose. The abscissa is per cent of mutants induced.

Novick: I see. You start out, then—

Freese: I start out with no mutants induced.

Novick: Oh, you treat *r* and get *r*+, *r*+ to *r*?

Freese: I go from *r*+ to *r*.

Auerbach: Don't you get any spontaneous mutants?

Freese: The spontaneous mutants are negligible as soon as I plot the first point on this curve.

Auerbach: I see. They couldn't explain this discrepancy, though.

Freese: Oh, no.

Benzer: You can have some $r+$ in a plaque and it may not look mottled. I wonder whether you picked the r plaques and replated them to see if they contained a minority?

Freese: No. This analysis has not been done in the detail to rule out this possibility, but, since we get the straight line in this plot—

Benzer: That factor might tend to make the number even higher. If some of what appear to be pure r plaques actually contain some $r+$ particles, the number of mottled would tend to be underestimated.

Goodgal: Might it mean that the curve is the result of compensation, and the straight line doesn't mean anything.

Freese: No. The straight line does mean something, but it could be that the whole straight line has to be shifted.

Glass: Would you mind stating your final conclusion again? I'm not sure that I followed the derivation of that from the data.

Freese: One can draw two conclusions: first, one would expect that the longer one treats phages by a mutagen, the more pure mutants one should get, because, occasionally, one kills that strand which does not have the r mutation in it. That is clearly indicated by this curve. The second question is whether, by extrapolating to zero treatment, one finds only mottled plaques or whether one still gets some r plaques. Although the examination has not been done in the necessary detail, I think all our observations indicate that even at zero time, you would not get only mottled plaques but also r plaques.

There is one objection. One should really pick the plaques for all points along the curve and replate them, even those which look like pure r plaques. We have done this for a few points and then found in agreement with the above statement that most r plaques contained only r phages and not a mixture of both wild type and r phages.

Auerbach: That could account for your missing 30 per cent. If that were so, it would mean you missed 30 per cent.

Freese: Yes. I don't think that is very likely.

Benzer: The curve drops rather rapidly. Does that imply that a hit somewhere else is preventing development of one of the chains?

Freese: Yes, I would say so.

Benzer: It goes more rapidly than you would expect if it required another r hit to knock out a mottled plaque.

Freese: Yes.

Auerbach: This problem comes up in Kaudewitz's paper (38), which I found somewhat puzzling. It is the only paper which I know on treatment of bacteria with nitrous acid. He tested

for forward mutations by the replication method, and he did obtain mutations. While Vielmetter in phage got a one-hit curve for mutation, which is all right on his hypothesis that lethal hits are very frequent, Kaudewitz got a two-hit curve. This agrees with the two-strandedness of DNA, but then he should have had mosaics and he had none. Altogether he had 539 mutants at survival rates ranging from 10^{-2} to 10^{-4}. None of these was a mosaic. Then he thought of the objection that Dr. Benzer raised just now, namely that he might have missed small non-mutant sectors. So he took 20 mutants that had been obtained with low doses, where lethal hits on one strand accompanying a mutation are least likely, and tested them fully by making suspensions and plating them. None of them was a mosaic. Actually, this result would need to be corrected for spontaneous mutants, which may be nonmosaic; for one of his 20 fully tested mutants had arisen without treatment, and on the basis of the frequency of mutations in the untreated series, one would expect that at least 6 of the others were also of spontaneous origin; so this would leave only about 13. Still, it is puzzling that none of these was mosaic. I should add that there is nothing in the method that would obscure the occurrence of mosaics; for with the same method P^{32} had been found to produce many mosaics in experiments likewise carried out by the Tübingen group. In those experiments, about 30 per cent mosaics had been obtained at a survival of 10^{-4}.

Altogether then, the experiments on bacteria are difficult to interpret. The two-hit curves for mutation and killing suggest that the bacteria were binucleate. Kaudewitz says they were not, but I cannot see how he can feel so sure about it. He draws the conclusion from observations by Witkin and from his own experiments with P^{32}, but in all these experiments there had been different conditions of culturing and treatment. However, even if the bacteria in the nitrous acid experiments were binucleate, where were the expected mosaics? In any case, these data cannot be taken as showing that nitrous acid in bacteria acts in the same way as in phage, namely, by deaminating a base in one of the two strands of DNA.

Zamenhof: In our paper (84) on the induction of mutation in bacteria by nitrous acid, we also did not find mosaics. But I want to refer here to the work on heating (85), where bacterial spores, immediately after heating and without an intermediate cultivation, were plated, and one obvious way one could detect auxotrophs in this case would be if the second strand in DNA and the second or third and fourth nuclei were all destroyed. Up to 10 per cent of auxotrophs were discovered

that way, at survival of the order of 10^{-7}. In this case, it was most probable that at that low survival rate, all the second strands and other nuclei have to be destroyed.

Auerbach: That is very drastic treatment. In Kaudewitz' experiments, survival in some series was 30 per cent or higher.

Zamenhof: I don't know about this case, but there are situations where one can be sure that all that remains is just one strand of DNA, because otherwise, one could not detect auxotrophs at all.

Auerbach: If you work on the model that it acts on one strand originally, but couldn't heat act on the whole double helix?

Zamenhof: Yes, heat does, but it would not produce this particular auxotroph in the second strand. What do you say, Josh? Wouldn't it be that, if you detect an auxotroph without intermediate cultivation, all the second strands of DNA and the other nuclei must have been destroyed?

Lederberg: Well, destroyed, or not giving a large clone. I agree with the statement though.

Auerbach: But couldn't there be treatments which affect both strands at the same level, with heat?

Zamenhof: That is unlikely to be a rule. It could be an exception, a very rare exception. If you have 10 per cent auxotrophs it would be very unlikely that you hit both spots on two strands in all 10 per cent.

Neel: These are not single phage infections of bacterium; each bacterium is infected by multiple phages, is it not?

Freese: No, by single phages.

Lederberg: The bacterium is far too complex a genetic entity to do experiments on mutagenesis with it. I don't think we have enough information on the genetic structure of the bacterium to go into the chemistry of mutagens with it. Quite apart from the steps between putting the chemical into the cell and the time it reaches the DNA, the organization of DNA itself is certainly very complicated. You talk about double-stranded DNA; it must be single-stranded at certain stages. I have always been puzzled by the fact that with every dose, with everything we have tried in inducing mutations, we always get a mixture of mosaics and intact mutants. I think this has been everybody's experience. You don't know that you don't have an equally large number of mixed colonies, that you don't pick up as mutants in your system, Stephen.

Zamenhof: That is true.

Neel: But if they were auxotrophs, they would be overgrown by—

Lederberg: I agree, but, in addition to the ones that are intact, by

my own experience and every other experience that I know of, at any rate, there are many mottled or mixed colonies as well.

Zamenhof: Oh, yes, among the remaining colonies which could not be picked as auxotrophs.

Lederberg: Well, I think, too, there is not that much consistency in Witkin's data in this respect (80). I agree with you that the matter is extremely puzzling. I think that it reflects considerable ignorance as to what the details of genetic structure of bacterium are. You have a bacterium in different states of division at the time of the treatment, and I think we can assume that some DNA will be well compounded, that it will be in the double strand. We just do not know how homogeneous the different specimens are in terms of the state of the DNA at the time of treatment. Spores are, perhaps, a more favorable material than *E. coli* because it is at least likely that they are mainly uninucleate, whereas, in the case of an *E. coli* suspension, you have a very different situation. One cell is very different from the next one.

Auerbach: I had the same problem as Dr. Zamenhof when I scored spontaneous mutations in Neurospora, as I described yesterday. I felt the same way; we just know too little about the nature of the genetic material in Neurospora. These were dried spores and spontaneous mutations, so there was no drastic treatment.

Magni: I want to go back to Dr. Auerbach's last point on the two-hit inactivation curves with nitrous acid in bacteria. We also found two-hit inactivation curves with the same mutagen in resting haploid yeast cells which are certainly not binucleate.

Auerbach: How much does this depend on conditions? I am thinking of the work they do in the Hammersmith Hospital in London, particularly Alper (2), who finds in bacteria that the target number depends not only on conditions of treatment of bacteria, but also, for instance, on the plating medium; so that it is difficult to know what the target number is.

Magni: This is true, according to my experience for bacteria, but not true for yeast. We never found a multihit curve on haploid yeast with X-rays, changing the plating conditions.

Auerbach: After nitrous acid, I want to go on to experiments with purine and pyrimidine analogues. Here I have a very specific question to ask, but I should like to preface it by a few points that puzzle me. There is a presumption, a very plausible one, that bromouracil acts by being incorporated into DNA instead of thymine. There are a number of points which make me doubtful of this.

One is that, until yesterday, I thought that bromouracil was the only base analogue that is incorporated, but now I have heard that 2-aminopurine is also incorporated. But certainly, one does not assume that caffeine or azaserine is incorporated in bacterial DNA? Is it so sure—and this is the first question I wanted to ask—is it really quite sure that *all* the bromouracil goes into DNA? I read a note by Dunn in which he states that some thymine goes into RNA (46). Could this not apply also to bromouracil, and if so, could not the amount incorporated be different in bacteria that have been infected with phage? My first reason for doubting the incorporation hypothesis is, therefore, that many excellent mutagens are not incorporated, and that if a small amount of bromouracil remains outside the DNA, it might be this and not the incorporated one that is mutagenic.

Secondly, in Litman's experiments (45), there was very little correlation between the amount of bromouracil incorporated and the induced mutation frequency. In fact, even when 100 per cent of the thymine had been replaced by bromouracil, there still were rather few mutations. I expect one could say that only a certain probability exists of an incorporated molecule producing a mutation, but there should still be a correlation between the amount incorporated and the proportion of mutants.

Freese: No, not necessarily. If the mutation arises by a mistake in incorporation and not by a mistake in duplication, there need be no correlation between the amount of incorporation and the extent of mutation.

Auerbach: A mistake? But if a hundred mistakes are made?

Freese: I mean a mistake in incorporation (23) as one by which the bromouracil is, by mistake, incorporated by pairing with guanine instead of adenine; one could think of the possibility that very little bromouracil gets incorporated and yet many mutations are made by the incorporation of the bromouracil in the place of cytosine.

Auerbach: Has it ever been tested? For instance, Dr. Zamenhof has strains which differ in the extent to which they incorporate bromouracil, but has it ever been tested whether they also differ in mutation frequency (88)?

Zamenhof: We have not tested it quantitatively.

Auerbach: Dr. Freese, in one of your experiments, for a very specific question, you mixed thymine with bromouracil in order to see whether this affected specificity (25), but did this alter the over-all mutation frequency?

Zamenhof: We have not tested it. But Dr. Greer in our laboratory

found that the 5-bromouracil-induced mutant frequency does not increase when bacteria are subjected to 20 cycles of 5-bromouracil incorporation and replacement by thymine; furthermore, when bacteria are grown in 5-bromouracil and tested on media containing thymine only, the induced mutant frequency was the same as when tested on medium containing 5-bromouracil.

Auerbach: I mean, when you added more thymine, did you get fewer mutations?

Benzer: It is technically difficult to distinguish between mutations produced by the bromouracil going in or by the bromouracil being in, because you get so many mutations during the incorporations step.

Auerbach: I mean something much more radical. Could not bromouracil act from outside the DNA? Certainly, caffeine must do so, but could not bromouracil do the same? Must it act by incorporation?

Freese: There is no evidence that proves that it acts by incorporation.

Auerbach: Except that it is the most plausible interpretation.

Freese: Yes, this is the most plausible.

Goodgal: Just one comment. You don't really mean that concentration has no effect on the mutation rate?

Freese: I didn't say that. I said the amount of incorporation.

Goodgal: Yes, but then there must be some level at which the frequency would depend on the amount of incorporation, isn't there?

Freese: Let me tell you about an experiment which one could think of, which one cannot do at the present time, but may be able to do in the future, with DNA polymerase. It would be to add both thymine and bromouracil and extremely little cytosine. In this case, you would preferentially make mistakes by which the bromouracil is incorporated in place of the cytosine, and you would prevent the incorporation of the bromouracil by adding much thymine. In this case, you should get many mutations if you could prove that you have mutations in the DNA, but you should not get much incorporation.

Zamenhof: I could suggest an experiment which would follow along the lines that you have mentioned. If one incorporates 5-bromouracil into DNA having transforming activity (19), and then subject it to Meselson's density gradient centrifugation (72), one eventually comes out with three fractions (peaks); that is, one corresponding to molecules without any bromouracil, another one with only one strand labeled, and the third one with two strands labeled. Now, the specific transforming activity of each of these peaks is practically the same. It makes no difference for the transforming activity whether there is

bromouracil at all, whether it is in one strand, or whether there is bromouracil in both strands of DNA. But if one could test whether there would be more mutants from DNA of the third peak than of the first peak, this would answer your question. That would be a direct experiment.

Goldstein: When you have DNA which has incorporated bromouracil very heavily in place of thymine, that incorporated bromouracil must pair adenine against it quite normally. The evidence for that is clear —otherwise you would have only dead organisms. In other words, DNA can have half or more of its thymine replaced by bromouracil, and yet it is still a functional DNA. The proportion of mutant genes to total genes is extremely small. This suggests to me that the mistakes are made subsequent to the incorporation.

Auerbach: How does one explain the fact that Rose Litman found that the bromouracil could be counteracted by serine in the medium?

Freese: I think the media are so complicated that we can't be sure.

Auerbach: Would smaller amounts of bromouracil be incorporated, or would something in the metabolism of the bacterium counteract its effect in such medium?

Freese: The sulfanilamide medium which she used is so complex that I would not draw any conclusion about the detailed manner in which the mutations arose.

Auerbach: So one should conclude that the most plausible explanation is that bromouracil acts by being incorporated, but it is not proved that it acts so. Now, how is 2-aminopurine incorporated?

Freese: We have measured in collaboration with Dr. Gottschling the incorporation of 2-aminopurine in phage T4 DNA and found that about one in 3000 adenine bases is replaced by—well, I shouldn't say that. I should say that the amount of incorporation of 2-aminopurine is 1/3000 of that of adenine into the DNA.

Goodgal: You have your nearest-neighbor analysis?

Freese: Oh, no.

Zamenhof: What is the possible error in determination of that small amount?

Freese: This has been done with radioactive purine. We have dialyzed the DNA for seven days and then digested it and found the aminopurine, whatever that treatment means. I cannot exclude that an amino-purine base has occasionally been attached to the DNA molecule without being incorporated, but that does not seem very likely.

Auerbach: Then how do you explain that aminopurine is so ex-

traordinarily more efficient per amount incorporated than bromouracil?

Freese: Let me start with bromouracil. I want to say that all we tried to do was to make a correlation between, in this case, a chemical theory and genetic observations. In order to prove our point completely we would have to make a sequence analysis of normal and mutant DNA. But this is completely impossible at the present time.

Figure 16 shows the normal pairing between adenine and bromouracil. Occasionally, 5-bromouracil may either lose the hydrogen on the 3-position, which means that it may be ionized or that it may undergo a tautomeric shift, in which case it loses this hydrogen and attaches one at the 4-position. In either of these two cases, it can pair with guanine, when it is ionized by two hydrogen bonds, or when it has undergone a tautomeric shift, with three hydrogen bonds, the same hydrogen bonds which we observe in a guanine-cytosine pair. This pairing should be perfectly normal with respect to the C-N distances and angles.

Glass: Why can't ordinary uracil do that, then?

Benzer: Ordinary uracil can't get in because it doesn't have a group in the 5-position that the enzyme requires.

Freese: It is very critical to have the right molecule at the 5-position. Bromine has about the same Van der Weels radius as the methyl group in thymine, and this is extremely important. If you take iodouracil or chlorouracil, it does not get incorporated to the same extent, and fluorouracil does not get incorporated, to any measurable amount, into DNA.

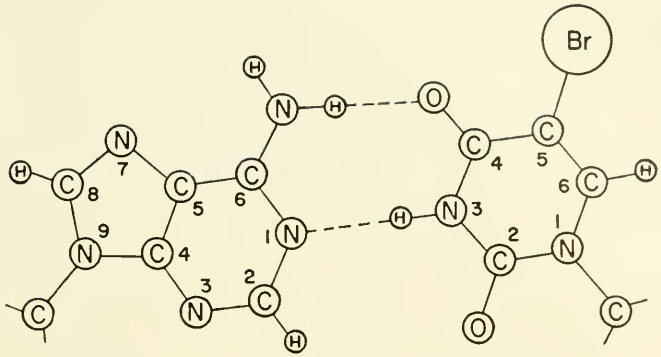
Auerbach: Do the others produce mutations—the chlorouracil, for instance?

Freese: Iodouracil does, but I'm not sure about chlorouracil. The question is, why is bromouracil mutagenic? We know that bromine is more electronegative than the carbon in the methyl group, and therefore pulls the electrons out of the ring. In some way, not understood in detail, the hydrogen at the 3-position thereby becomes more labile than it is in thymine. I think I should really not say more about this because it is all conjecture.

One way to prove this view would be to employ compounds with different groups at the 5-position which differ in their electronegativity but do not differ very much in size (this is obviously quite difficult to do); then one could see whether the frequency of mutations increases with the electronegativity.

Auerbach: Is this what you call mutagenesis by replication?

Freese: Whenever the mistaken base pair bromouracil-guanine is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1a
Adenine
(normal amino - state)
5 - Bromouracil
(normal keto - state)

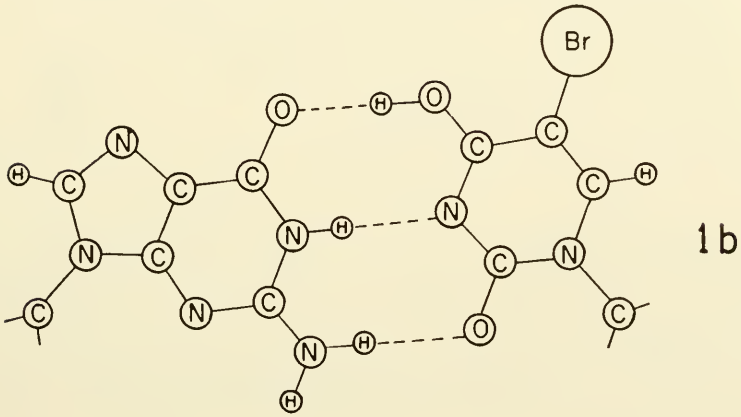
Br
1b
Guanine
(normal amino - state)
5 - Bromouracil
(rare enol - state)

Figure 16.

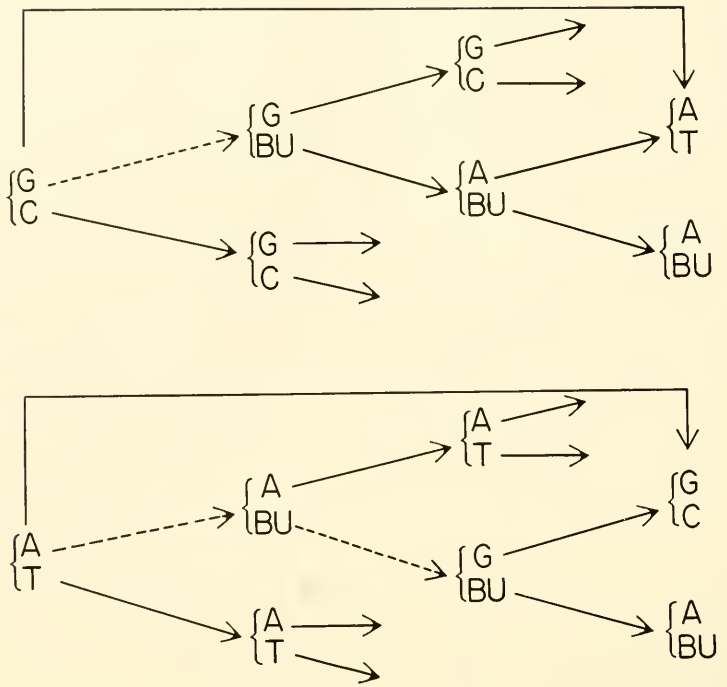

Figure 17.

incorporated into DNA we should get a base pair change. This can arise in two ways (Fig. 17). Either bromouracil, as a deoxynucleotide triphosphate, pairs with a guanine base already present in DNA, and the bromouracil nucleotide then gets incorporated into the DNA. Later, when this DNA pair duplicates again, BU chooses its normal complementary base which is adenine. In this way a "mistake in incorporation" occurs.

The other possibility is that bromouracil first gets incorporated by pairing with adenine and then when the DNA duplicates again, this bromouracil pairs by mistake with guanine, in which case we get a mistake in replication.

Auerbach: But you had another one where it goes—

Freese: No, these are the two possibilties. In the one case, when the bromouracil makes a mistake while it goes in, the original guanine-cytosine pair gets mutated, eventually, into an adenine-thymine pair, while, when bromouracil is first incorporated and then makes the mistake in pairing, we go in the opposite direction. Thus we can change both base pairs.

Neel: Are you postulating, then, that bromouracil has properties such that no matter what its original pairing, it is by reason of its chemical structure more apt to pair with the alternate possibility than the normal molecule would in that position?

Freese: That's right. Actually, it is not only able to make this mistake more frequently than thymine, but more frequently than the whole background of spontaneous mutations. Figure 18 shows

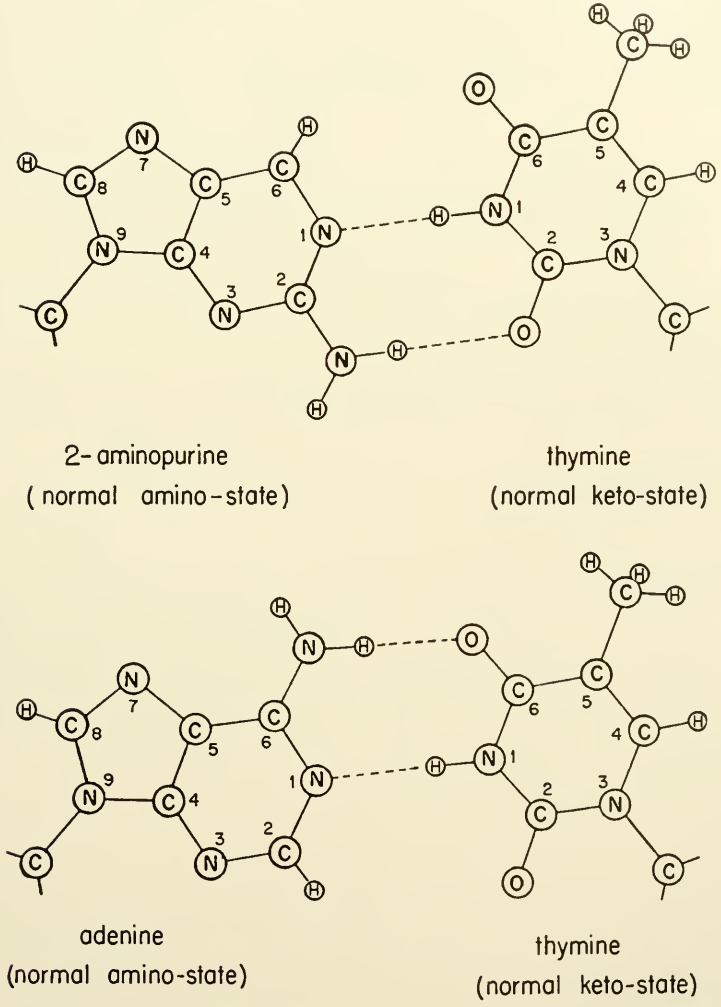

Figure 18.

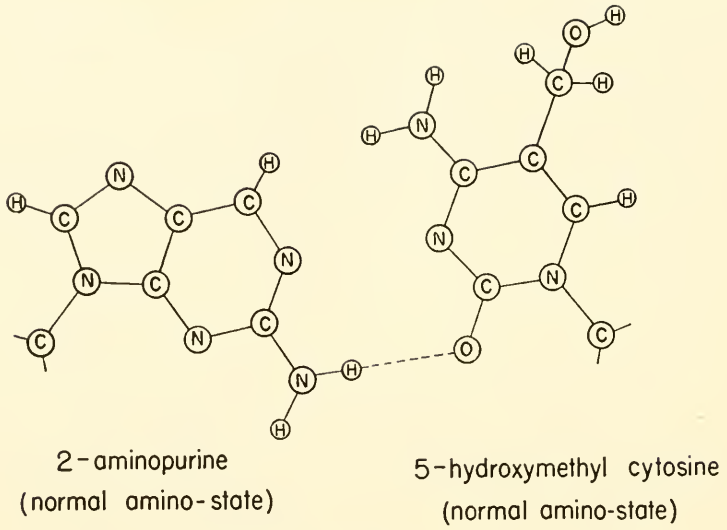

Figure 19.

2-aminopurine. Normally it should be able to pair at these two positions with thymine. The two hydrogen-bonds are the same as one observes in the pairing between guanine and cytosine; so, again, the distances and angles should be proper to allow pairing.

The question was why is 2-aminopurine so much more mutagenic than 5-bromouracil with so little 2-aminopurine incorporated.

Auerbach: About 3000 times as much, from what you say.

Freese: Yes. The explanation is given in Figure 19. While bromouracil can make a mistake and pair with guanine only after it has lost one hydrogen, as I showed before, 2-aminopurine can make one hydrogen bond when both of the molecules, aminopurine and cytosine, are in their normal tautomeric state; in other words, this one hydrogen bonding should occur as frequently as the two hydrogen bondings which 2-aminopurine makes with thymine. The only difference is that this bond does not force the base pair into the right distance and angle, but the base can swing about. Occasionally, however, the base will have the right distance and can get incorporated into DNA. The frequency with which this occurs should be much higher than the frequency with which a tautomeric shift occurs for 5-bromouracil.

Auerbach: Can you estimate the probability for aminopurine?

Freese: No. The argument is the following: The frequency of tauto-

meric shifts is really not known. If we take the frequency with which bromouracil induces mutations we can estimate that the ratio of bromouracil, being in the normal state to the one being in the tautomeric form, might be of the order of, let's say, 10^{-7}. In contrast, the 2-aminopurine-cytosine base pair might have the right distance and angle in perhaps 10^{-3} of the cases in which such a pair is formed.

In the case of adenine (Fig. 18) you have the amino group in the 6-position. You have to look at the molecular model. The two amino groups interfere; there is no hydrogen bond possible between two amino groups, and there are no hydrogen bonds possible otherwise.

Benzer: What do you expect with 2,-6-diaminopurine?

Freese: I would say that the same argument applies as for 2-aminopurine; 2,-6-diaminopurine can also pair with cytosine by a hydrogen bond at the 2-position.

Benzer: No, it can't pair in the 6-position.

Freese: The 6-position should interfere, and therefore the effect of 2,-6-diaminopurine should be less than that of 2-aminopurine. But it should be mutagenic.

Benzer: It shouldn't work at all. It is impossible to form this base pair.

Freese: No, I mean this base pair can be formed.

Benzer: You can't form the complementary pair of bases. You have two amino groups knocking head on.

Freese: But I'm not making any other base pair at the 6-position.

Benzer: You won't be able to make a proper base pair with two amino groups.

Freese: Why not? I can move the molecules sideways. The question is only whether it gets incorporated. I certainly can make that one hydrogen bond in the 2-position.

Auerbach: I wanted to say something similar. In Vielmetter's scheme, hypoxanthine is an intermediate. That, I think, is incorporated. But does it produce mutations?

Freese: No, hypoxanthine does not get incorporated into DNA.

Auerbach: I thought I saw in one of Kornberg's papers that it was incorporated.

Freese: Oh, yes, in Kornberg's papers it is incorporated. That is quite correct. He has to make artificially the hypoxanthine deoxynucleoside triphosphate.

Auerbach: How do you make that? Does he feed it to a phage?

Freese: No, you can't get any nucleotides into a bacterium. This

hypoxyanthine deoxynucleoside triphosphate is not made in a bacterium, so you don't find any inosine in DNA.

Auerbach: Is there any theory to account for the mutagenic effect of compounds that are not incorporated?

Freese: I think most clearcut is the observation for azaserine, which we know interferes with the purine synthesis; caffeine probably does the same. Thus it may be that their mutagenic effect is due to the distortion of the normal purine metabolism; for example, due to an abnormal ratio of adenine and guanine DNA precursors.

Auerbach: That would explain the antimutagenic effect of adenosine riboside.

Freese: Yes, that would explain the antimutagenic effect at the same time. Now the difficulty is that some experiments have been done, I think by Magazanik, where they have tried to influence the direct DNA precursor pool by adding a purine analogue or by using mutants, and, to their surprise, they found that whenever you have a condition under which the DNA can duplicate, you find approximately the same ratio for guanine and adenine as you find in normal cells.

Auerbach: This is also true for caffeine. However, when 8-ethoxycaffeine breaks chromosomes, it does so at a stage when DNA replication is already finished.

Freese: Is that proven?

Auerbach: Well, Kihlman (40) found it.

Freese: There is the possibility that the bulk of the DNA duplicates at a certain time and a few DNA linkages are made later, and that is sufficient—

Auerbach: No, at the end of interphase in plants, where I think the DNA is—

Freese: Then, in some cases, you have new DNA synthesis already, so I think one has to examine this very carefully.

Auerbach: The antimutagenic effect of purine ribosides was very weak in Kihlman's (41) experiments. He had some, but very little, not comparable at all with Novick's experiments.

Zamenhof: I would like to discuss in a few words another mutagen —which is new because it is so old that it became unfashionable— deuterium.

Something like seventeen years ago, with Dr. Demerec, we studied mutagenicity of heavy water in Drosophila (89) and did not find any. But now the techniques of microbial genetics have progressed so far that we thought this should be reinvestigated. Miss De Giovanni in our laboratory has now found that deuterium oxide *is* mutagenic (12,

13); the range of increase of mutation rates was from about ten times to a hundred times, using various markers such as streptomycin dependence, thymine dependence, streptomycin sensitivity, and so on. Recently, this mutagencity was also found in phage by Stent's group.

Our thinking was first in terms of the methyl group on the fifth carbon of the uracil ring; since we found that substitution of this methyl group by bromine produced mutations (87), we thought that making this group slightly heavier by replacing hydrogen by deuterium would also have an effect.

I want to say right away that we have no proof whatsoever that deuterium acts through DNA. It may affect other parts of the cell, as Dr. Auerbach mentioned. Still, it is tempting to speculate on this subject, and there is some evidence that if deuterium goes into the molecule of thymine, it goes also to the methyl group.

We analyzed such DNA and, indeed, found that in the thymine of DNA from bacteria grown on heavy water, out of four nonlabile hydrogens, two were replaced by deuterium. Then we took this thymine, which had the deuterium, and, using a thymine requiring strain, incorporated it again into DNA, but only as thymine, to see whether this procedure was mutagenic. It was completely nonmutagenic.

We investigated other bases, purines and cytosine, which had deuterium in the molecule, using strains which need this particular purine or pyrimidine and incorporate it into DNA. It turns out that there, too, the bases are not mutagenic. It seems that the only plausible conclusion would be that either mutagenicity is through a change of hydrogen bonds into deuterium bonds, or else mutagenicity of deuterium is not due to deuteration of DNA.

Auerbach: We will now leave the purines and pyrimidines and related substances. I should like to say a few words about the action of other mutagens, for which the relation to DNA is less obvious. There are first the alkylating agents, which for a long time were the best-known mutagens. They can be divided into chemical classes according to the effective alkylating group they carry. The best known of these are: the chloroethyl group which occurs in mustards; the ethylene imine or ethylene oxide group; and the methane sulphonate group.

In higher organisms, there is no way of knowing whether these substances act directly by alkylation of DNA or more indirectly. One thing emerged from work on Drosophila. There it could be shown that at least one of these substances, mustard gas, acts as "hit poison"

in the sense of the German biophysicist Jordan; that is, it produces its effects by discrete, more or less random events in the genetic material, similar to X-rays. This was proved by dose-effect curves. For X-rays, it can be shown that individual "hits" are responsible for mutations and chromosome breaks, because with increasing dose the frequency of chomosome rearrangements, for instance translocations, increases approximately as the square of the frequency of lethals. This is so because translocations require two independently produced breaks. Dose-effect curves for chemical mutagens seem quite unreliable to me because they obviously must be distorted by factors such as penetration, diffusion, etc. But what one can do in an organism like Drosophila is to plot one effect against the other. When this was done (56) for mustard gas it was found that translocation frequency increased almost exactly as the square of lethal frequency. This indicates that two independent events, one of which is sufficient to produce a lethal, are required for producing a translocation. By this criterium, mustard gas has been shown to act as "hit-poison."

How exactly alkylating agents act cannot be derived from work with higher organisms. Recently, Loveless (47) from the Chester-Beatty Institute in London reported that one of them—ethylmethane sulfonate—produces mutations in phages *in vitro*. Ethyl sulfate had the same effect. This was quite a breakthrough because until recently it had not seemed possible to make phages mutate by treating them *in vitro*. Then Krieg in Oak Ridge managed to do this with ultraviolet radiation, and this was followed by Loveless' success with chemicals.

Green in Oak Ridge confirmed Loveless' results, and he further established that the mutations are not produced by ethylmethane sulfonate that is carried passively into the bacterium and acts there during phage formation. He showed this by infecting bacteria with a mixture of treated and untreated phage, and all the mutations occurred in the treated one. This, as far as I know, is the only evidence for an alkylating agent acting directly on DNA. Dr. Freese said that he has an explanation for this effect.

Freese: I think it is important to point out that ethylmethane sulfonate is a monofunctional ethylating agent; only the ethyl group can react whereas the methyl group is firmly attached to the sulfate. We have worked with a similar agent, ethylethane sulfonate, in which the ethyl group is again the active one. This has been done mainly by Mr. Bautz in our laboratory, and we have found the following (6):

First, we made a theoretical consideration; one knows, from experiments by Reiner and Zamenhof (60) and by Lawley (43), that diethyl

sulfate or dimethyl sulfate reacts mainly with the 7-position of guanine (see Fig. 20). This automatically induces a quaternary nitrogen at this place that is positively charged. But this molecule is in resonance with another state, in which the charge is not on this nitrogen but on the 9-position nitrogen on which the ribose phosphate is attached.

It is clear that the positive charge makes this molecule rather unstable and, subsequent to the attachment of the ethyl group, either the ethyl group itself can separate off again or the sugar-base bond can break.

To test this idea we first dialyzed DNA in order to remove all the nucleotides and bases which contaminated it, then added diethyl sulfate to the dialysis bag, incubated it at 37° for two days, and then examined the dialysate. Indeed, we found that the dialysate contained 7-ethyl guanine. The amount of ethyl guanine liberated is about 0.5 per cent of the guanine present in the DNA, in this particular treatment.

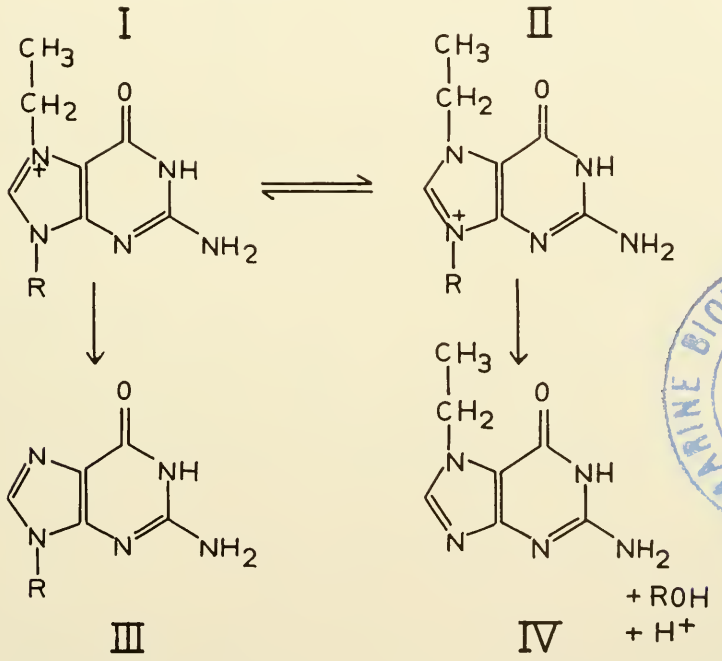

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Figure 20. *Proposed mechanism* of depurination by ethylation. I and II: mesomeric states of 7-ethyldeoxyguanosine. IV: removal of R- caused by state II. R- = deoxyribosyl-.

Lederberg: Is that the only thing that comes off?

Freese: Yes, this is the only thing that comes off. There is no adenine that we can observe. The amount of adenine must be less, or the ratio of adenine to guanine coming off must be less than 1 to 50.

Zamenhof: Adenine is also alkylated (60).

Freese: Yes, adenine is alkylated. But Lawley has shown that it is preferentially alkylated in the 1- and the 3-position.

Benzer: How much does this treatment correspond to in terms of the conditions used for mutagenesis?

Freese: That's hard to judge because, first of all, we didn't do it on phage DNA but on herring sperm DNA, and secondly, we used diethyl sulfate, which reacts much stronger and much faster, instead of ethylethane sulfate. But it is an ethylating agent.

Auerbach: Is it mutagenic?

Freese: Yes, diethyl sulfate is mutagenic, but it is very difficult to work with as a mutagenic agent because it reacts so fast with water.

Auerbach: Loveless got mutations.

Freese: Yes. It does induce mutations, but it is harder to work quantitatively with it. So it is difficult to compare quantitatively the chemical and the mutagenic experiments. That, naturally, has to be done. But I think it is clear that there is efficient depurination; other experiments by the Zamenhof group with bacteria and by ourselves with phages have shown that depurination—in Zamenhof's case with high temperatures (90) and in our case with low pH (23)—causes mutations. It is likely that the main mutagenic effect of at least this alkylating agent is due to the occasional removal of guanine from DNA (31, 48).

Auerbach: Would that mean that it always produces deficiencies when the purine goes off?

Freese: This is another point. At the time we did the experiment with the lower pH treatment, we didn't know what kind of changes would be produced, but it turns out that most of the mutants we get are quite similar to mutants induced, or behave in the same way as the mutants induced, by base analogues (24); in other words, they are presumably due to changes from AT to GC, and vice versa.

Auerbach: How would that come about?

Freese: It could have been that the gap prevented the DNA to duplicate across this place or it could have been that the gap causes a loop and a larger deletion. But the results indicate that DNA does duplicate across the gap and just incorporates one of the four bases opposite to the gap. Interestingly enough, the bases which seem to be

incorporated preferentially across the gap are the pyrimidines, because we apparently get more of the above mentioned mutagenic transitions than the changes in which a purine is replaced by a pyrimidine, or vice versa.

Auerbach: I'm sorry, but what is the evidence that you got these changes? I have forgotten.

Freese: Because 70 per cent of the mutations we get in this case are inducible to revert by the base analogues bromouracil and 2-aminopurine.

Glass: I don't quite understand. Is the change on the charge of the nitrogen in position nine going to produce depurination? What makes the depurination take place?

Freese: Actually, you have to imagine that the positive charge is distributed over the imidazole ring, and whenever you have such a positive charge, these groups are more labile. That is just a chemical fact. It corresponds to our observation that the guanine goes off.

Glass: They fail to become attached to the deoxyribose?

Freese: No, this is DNA. You can take free DNA, which has the guanine in the DNA; treat it with diethyl sulfate, and then the guanine is removed from the DNA.

Neel: And you recover guanine in the experimental system?

Freese: You recover guanine in the dialysate.

Auerbach: And this is due to the bond with the sugar being broken?

Glass: Yes, that's what I meant.

Atwood: A few months ago, Krieg and Green had some preliminary experiments which suggested that mutations induced by ethylmethane sulfonate in phage were not reversible by that same agent. That is predicted from your model, is it not?

Freese: Right. We have also done experiments on the induction of reversions by ethylethane sulfonate, and we found that certain mutants which had been induced by aminopurine are much more inducible to revert than most of the mutants induced by bromouracil.

Zamenhof: Ernst, as you know, Dr. Greer in our laboratory found that heat depurinates under mutagenic conditions (32); would you agree that it is not completely impossible that the depurinated locus always reproduces as depurinated rather than picks up a wrong base? I think that Kornberg is now investigating whether or not deoxyribose triphosphate might participate rather than nucleoside triphosphate, that is, whether the former can stay naked, so to speak, after reproduction.

Freese: To stay naked all the time?

Zamenhof: Yes.

Auerbach: How can you reverse it, then?

Zamenhof: In certain cases, nucleoside triphosphate may again replace deoxyribose triphosphate. In other cases mutations may be nonreversible. We have several "deletions" produced by heat; these deletions may or may not be the result of depurination. Of course, you never can tell about the nature of a "deletion." "Deletion" usually means that the mutant never reverts. But this could be because the injury is very large or because the injury is too severe, as when the sugar is destroyed; in this last case, the DNA could never hold a purine again.

Auerbach: But if the purine is out, you couldn't put it in again, could you? You couldn't reverse the mutation.

Freese: I could even imagine that in the resting DNA, already the empty place is filled by something. We don't know of any enzymatic mechanism at the present time which would do that, but it is not impossible; you have the nucleotide phosphate here, and it is not impossible that there is some enzyme which hooks on the base at this place before the DNA duplicates. We don't know.

Benzer: Green and Krieg's experiments show that what you get is an activated phage, which is mutation-prone and continues to throw off mutants during replication (31).

Novick: Excuse me, but before you go ahead, would you explain your interpretation? What would you conclude from this?

Freese: I would conclude—and we have worked with other mutagens which agree with this picture—that these aminopurine induced mutants which are especially highly inducible to revert by EES have a guanine-cytosine pair, and most bromouracil induced mutants have an adenine-thymine pair at the mutant site. This we conclude from the already mentioned chemical observation that guanine is attacked preferentially.

Lederberg: There is one point in the chemistry on which I am not clear. You referred to the specificity of this agent in removing guanine. Was there a comparable specificity in the ethylation itself? You referred to other products of ethylation which are not unstable, which don't give you—

Freese: Well, here, I can only refer to the experiments by Zamenhof (60) and Lawley (43), who have shown, with methylating agents, that the preferential effect on nucleic acid bases is the one of alkylation of the 7 group of guanine. There is also an alkylation of the 1 and the 2-position of adenine, and probably the 1-position of cytosine, but this is much less frequent. We have two more arguments which indi-

cate that the alkylation of guanine actually gives the main mutagenic effect.

If this is true, more and more guanine should be removed from DNA when we incubate the treated DNA, let's say, at 37°. Therefore, we treated phages for different times with ethylethane sulfonate and determined their survival after subsequent incubation for different times at 37° C. When we plated the phages 12 or 24 hours after the treatment, we found indeed that the phage titer had gone down appreciably, and the more the longer the original treatment lasted.

Lederberg: What do you visualize happens during this incubation period?

Freese: What I visualize is that the ethyl groups get first attached to guanine.

Lederberg: That happens during the treatment?

Freese: Yes, that happens during the treatment, which is short. Then, when the DNA duplicates right away, when you plate right away, some ethylated guanine gets removed with a certain probability, either during DNA duplication or before. I don't know why we get such a strong inactivation right away, but when we incubate the free phages at 37°, more and more of the guanines get removed from the DNA, and therefore more phages are killed.

Auerbach: What about mutations?

Freese: The frequency of *r* and mottled plaques increases by about a factor 2 after 24 hours of incubation.

Auerbach: If I may commit the mistake against which I always warn and extrapolate from phage to higher organisms, I should like to point to a very similar phenomenon in Neurospora or bacteria that have been treated with certain mutagens. When Dr. Kølmark and I treated Neurospora conidia with diepoxybutane (42), we found quite by accident that if one treats the spores, washes them thoroughly, and then just lets them stand, there is a very considerable increase in mutation frequency up to 10 hours or more. There was no concomitant decrease in viability, but I don't think that killing in these experiments has anything to do with mutation. Szybalski (71) found the same for bacteria that had been treated with TEM. What we found difficult to understand in our experiments was that this delayed effect was interrupted by plating, although plating was done on minimal medium without supplements where there should be no growth theoretically, just as in the water in which storing had taken place. However, in practice there always is some leakage and this perhaps stops the delayed effect of diepoxybutane.

Dr. Freese, did you find that the degree of aeration had an influence in your experiments? In one of my experiments, I kept part of the stored suspension violently agitated, and this seemed to destroy most of the delayed effect.

Freese: I think here one has to be careful. We remove the ethylethane sulfonate by adding thiosulfate, which reacts very strongly with the ethylethane sulfonate. Some people don't remove the alkylating agent after they have treated, and then you don't really know when the treatment ends.

Auerbach: Ordinary washing with centrifugation, even 5 times, did not destroy the delayed effect.

Freese: I must say that in higher organisms there is, I believe, quite a strong possibility that at least many mutations are not due to the direct effect on DNA, but either to the effect on nucleotides or the effect on proteins. This is indicated also by the effect of the bifunctional agents, which can have two effects. They can either have the same one as we have regarded here, or they can cross-link two molecules.

Auerbach: No, no, there is no evidence that bifunctional agents are better chromosome-breakers. I'll come to this later on.

Zamenhof: Dr. Auerbach, did I understand you correctly that the period of storage after treatment and removal of alkylating agent before plating plays a role in the determination of the number of mutants?

Auerbach: Yes, for the adenine locus in Neurospora. In one experiment we got 10 times as many mutants after 4 hours of storing in water at 30° as in immediately plated spores.

Lederberg: From the second result with the AT, not being reversed by ethylethane sulfonate, do you then conclude that the substitution of T for A is spurious, and that the base pair that has been originally replaced was not an AT but was a GC?

Freese: Yes.

Lederberg: What is the other evidence to support that picture for bromouracil? Can you tie that together?

Freese: Well, we have done similar experiments with hydroxylamine—

Benzer: Excuse me, but before you leave the ethylethane sulfonate, what is your prediction as to the mutants that are produced by ethylethane sulfonate?

Freese: Taking the results of depurination by low pH and high temperature treatment, I would predict that of the order of at least

75 per cent of the mutants are due to transitions. That means they are due to base pair changes from AT to GC or vice versa, and, in fact, from GC to AT.

Benzer: What you have is a gap.

Freese: However, we would expect to get some changes in which a purine is replaced by a pyrimidine and vice versa.

Benzer: Shouldn't those be equally probable? You have a not-base, and it doesn't pair with anything.

Freese: I reason from the experimental facts which we have observed with the low pH treatment—

Benzer: But you are assuming that the other one works by the same mechanism. You should make a prediction on this thing by itself.

Freese: I can give you an explanation, which would be that there are more pyrimidine precursors than purine precursors, so that across a gap, pyrimidine gets easily incorporated into the DNA, and the purine does not. But this is only an explanation of the observations.

Lederberg: Does the ethylethane sulfonate also reverse the mutants?

Freese: No, this we haven't done so far. The reference here was to ethylmethane sulfonate for which Green has found that he could not induce the reversions of these mutants.

Novick: You find that EMS-induced mutations are not reversible?

Freese: By EMS.

Novick: What about BU?

Freese: We haven't done that so far.

Zamenhof: Also, heat-induced mutations are not reversed by heat. They may reverse spontaneously but not by heating.

Benzer: Is the prediction sufficiently flexible that it will fit any experimental result?

Freese: With respect to transitions and transversions, yes. I cannot predict whether you get more transitions or transversions, but when you look at the reverse mutations, you predict that since we attack the guanine, we get preferentially changes from GC to AT. As long as you stay in the framework of transitions, I make a prediction. But how many transitions and transversions, I wouldn't dare predict.

Goldstein: Why couldn't you predict that the ethylethane sulfonate mutations would only be reversed by aminopurine? If the result of the gap is to pair C, you don't have a mutation, because you have the same GC pair that you started with. If the effect of the gap is to pair G, you have CG in place of a GC, and this still should not be affected by bromouracil, according to your theory.

Lederberg: No, that is a mistake in the premise. To be consistent

with this statement bromouracil, in fact, does affect precisely the CG. But it does not give you the substitution of one for the other.

Freese: All I would say is that ethylethane sulfonate should preferentially attack the CG base pairs. What it makes out of them, I cannot predict.

Atwood: If you think that the Green experiments are sufficiently conclusive—I don't know whether they are—then you're not getting transversions.

Freese: He has not shown anything of this kind.

Atwood: If, in so far as you would get transversions instead of transitions, they would be reversible by the ethylmethane sulfonate.

Freese: What would be reversible?

Atwood: If you merely turn it around so that you now have a GC instead of a CG, this could be reversible by another transversion.

Freese: Right; only these changes could be reversible. But if you get the change from GC to TA, for example, which is also a transversion, it would not be reversible by EES.

Atwood: At least if you find, as he thinks he has found, that there is no reversibility, that means there is no transversion with EMS.

Zamenhof: I would like to comment on the possibility that purines methylated on the 7th nitrogen (60) are kept in the molecule and are reproduced. I do not think this is possible. We tried to incorporate 7-methyl guanine into DNA of purine-requiring mutants and were unsuccessful. The methylated compounds possibly could be made by the cell. There may exist enzymes for such synthesis, but there are no kinases to make triphosphates from 7-methyl nucleosides or from 7-alkyl nucleosides, with an alkyl group as big as mustard. It is sure that such an alkylated purine has to disappear. The final outcome of the mutation cannot be the same as the first step.

Freese: But I disagree here. You have only shown that the 7-methylated guanine, or whatever you have taken, cannot be incorporated into DNA. You have not shown that the 7-methylated guanine in DNA cannot duplicate.

Zamenhof: Well, this is not shown by the experiment I mentioned, but there are no known kinases which make such a triphosphate.

Freese: In the cell?

Lederberg: You're talking about two different things. Freese was suggesting that the ethylated guanine in the original molecule in which it is produced might persist in that form, or might necessarily or ultimately always give rise to a mistake in some of the subsequent copies of that original DNA. Therefore, every initial event will

ultimately give rise at least to a mottled plaque, because sometimes that copy of that original DNA will have made a mistake. But he would suggest that that original ethylated guanine cannot persist as such in that original maternal molecule. He never suggested that ethyl guanine ever replicates after two cycles, so you again get an ethylated guanine. That is what you are denying.

Zamenhof: No, I am not denying anything he said. On the contrary, I am trying to amplify it. We realize now how complex the mutation process may be. It is a multistep process. In the first step, alkylation occurs. In the second step, DNA may lose purines. In the third and final step, which is finally accepted, the wrong purine gets there to fill the gap. I'm just trying to amplify that the process of mutation may be a very complex, multistep process.

Auerbach: Does Dr. Zamenhof think that all the alkylating agents act at the 7-position?

Zamenhof: No, I didn't say that. But I am sure that none of the alkylating agents, especially mustard, can be accepted as such during the replication of alkylated DNA.

Auerbach: Oh, no, no! But it is always the guanine group which is attacked in DNA?

Zamenhof: We have evidence that it is predominantly guanine (60).

Lederberg: Let's clarify the remark, for the record, that the alkylating group must come off. I think what you mean is that it cannot be reproduced as such in further copies of that DNA.

Zamenhof: Yes, that's right.

Lederberg: That does not mean, as a chemical entity, the alkylated nucleotide may not persist as such as long as that original DNA molecule persists.

Zamenhof: Correct.

Neel: Could you show us the difference between one-armed, two-armed, and three-armed mustard?

Auerbach: Well, the nitrogen mustard that is most frequently used has two chloroethyl groups. There is also one with only one chloroethyl group, but this is a rather weak mutagen. Ethylene oxide is one-armed, and so is ethylene imine. Two ethylene oxides linked together give the two-armed diepoxybutane. TEM has three groups of ethylene imine and is three-armed. The cancer research workers find that only two-armed or three-armed compounds are carcinostatic, but for mutagenic action this simply is not true. There is not the

slightest doubt of this. Some one-armed chemicals are better mutagens than the corresponding two-armed ones (67).

For chromosome breakage this has hardly been tested until recently. It seemed possible that breakage of two strands might require cross-linkage, but there is no evidence for it. For instance, beta propiolactone and ethylene imine, both one-armed, are good chromosome breakers. However, in view of the findings of the cancer workers and of the connection between carcinostatic action and chromosome breakage, it seemed worth while to test this question properly.

Last year I had a co-worker, Professor Nakao, who did experiments to test whether bifunctional alkylating chemicals are relatively more effective than monofunctional ones in causing chromosome breakage. By relatively I mean relative to their ability to produce what we think are mainly gene mutations. Nakao used the two related compounds ethylene oxide and diepoxybutane for producing lethals and translocations in Drosophila. We were not interested in the absolute frequencies, which were definitely lower for ethylene oxide, probably because it is a very unstable compound. We were interested in the relative frequencies of translocations to lethals, for the former require chromosome breakage while most of the latter do not. The ratio of the two effects turned out to be exactly the same for the two compounds, so there was no evidence for an effect of cross-linkage on chromosome breakage. Nakao intends to test another pair of compounds in the same way.

Lederberg: Is that correlation really very good for carcinostasis or—

Auerbach: Professor Haddow of the Chester-Beatty Institute assured me of this.

Goldstein: I want to know whether you can use nitrous acid to solve this question of what exactly is going on, in the following way: If you start out by assuming your proposal begins with a GC, which, in the presence of this alkylating agent winds up as gap-C, you don't know what is pulled into the gap, so we can draw four possibilities.

One is that G is drawn into the gap, in which case we have no permanent mutation. Another is that C is drawn into the gap and we get CG. Or T is drawn into the gap and we then get TA. Or A is drawn into the gap and we then get AT.

You now treat the mutant with nitrous acid. Then CG should go to TA, which is not a reversion. TA should go to CG, which is not a reversion. Only AT should go to GC, which is a reversion. So one would conclude that if you can get reversions with nitrous acid, this must be what has happened. Is that reasonable or is it not?

Freese: Yes. We have done these experiments, not with nitrous acid and not yet for ethylethane sulfonate, but for the phages treated with lower pH, for which we assume that the purine goes off, so if we take the case in which the guanine goes off, we would have a similar situation. The base analogues, bromouracil and 2-aminopurine, apparently induce only changes from AT to GC and vice versa, so we could not induce two of the three reversions which you mentioned. We found that 70 per cent of the mutants could be induced to revert by these base analogues, so they are presumably due to a transition.

Auerbach: And could be reversed.

Freese: Could be reverted, yes. We really have to do these experiments now with the alkylating agents.

Lederberg: Could I revert to a little bit of an earlier question that I think was not fully answered; that is, how do you interpret this? What is the maximum frequency of nonmottled plaques that you eventually do get by holding your treated or your ethylethane sulfonate-treated phage? That went quite high, didn't it?

Freese: The total frequency of *r* and mottled plaques goes up to 1 per cent. The frequency of mottled over the sum of *r* and mottled—

Lederberg: Goes down?

Freese: Yes. You mean with ethylethane sulfonate?

Lederberg: Yes.

Freese: Without incubation it is very close to 1; in other words, most of the plaques are mottled initially. Later, it goes down.

Lederberg: But how far does it go down?

Freese: I don't recall that. I wouldn't like to talk too much about this because we haven't done enough on it.

Lederberg: In fact, though, with the conditions of your experiment, you get mottled plaques in two ways: either by delay in the mistake in replication, or by the double-strandedness of the DNA.

Freese: Yes, that's right. There I can refer to an experiment by Green and Krieg (31), in which they treated phages with ethylethane sulfonate and then made a single-burst experiment. This means they infect the bacteria singly and then distribute the infected bacteria in such a way that each tube receives only one infected bacterium. After these bacteria have lysed they plate the content of each tube and score the number of *r* mutants versus the number of wild-type mutants for each bacterium. They have found that mutants can apparently be produced in every DNA duplication, with equal probability.

Lederberg: You get mottled plaques on single burst?

Freese: You get *r* and wild types, but from the ratio of the two, you can conclude at what time the mutation was induced.

Lederberg: Yes, but there should also be a fraction of mottled plaques, which would be occurring in one of the existing generations, you see, at the time of plating. It should be a substantial fraction.

Freese: Since you got 200 phages out, that is not very substantial. I really don't know whether they have observed this.

Lederberg: That's only what? Six or seven or eight generations? Do I take it, though, that you're working at such levels of inactivation that you can assume that much of your phage inactivation is essentially in terms of the active strand rather than the single strand, but disregarding the mottling due to separation of the two strands?

Freese: I really don't know what the mottling is preferentially caused by in experiments with EMS or EES.

Lederberg: I'm worried about nonmottling.

Freese: I would say that the nonmottling probably means that one of the two DNA strands has been killed off.

Lederberg: Would you explain why the methyl agents are less active in producing phage? This was Loveless's report.

Freese: Yes, Loveless (47) found that the methylation is less mutagenic than the ethylation, and we have confirmed this using methylmethane sulfonate and ethylethane sulfonate. When one plots the frequency of mutants per viable phage versus the number of lethal hits—

Auerbach: Excuse me, but I don't know what you mean by "lethal hits." Inactivation?

Freese: The term "lethal hit" is derived from the Poisson distribution. A lethal hit is equal to the natural logarithm of the phage titer before versus the phage titer after treatment. In most cases the number of lethal hits increases linearly with the time of treatment but sometimes it doesn't; then the lethal hits are a better measure for the extent of the chemical reactions than the time of treatment.

We find that the frequency of *r* mutants increases with increasing lethal hits about five times faster with EES than with MMS. When we do the same for reverse mutations we find that the frequency of reversions is also increasing much faster with ethylethane sulfonate than with methylmethane sulfonate.

Neel: What is the relative frequency of forward and back mutations?

Freese: About 10^{-3} to 10^{-4} for highly inducible mutants. This clearly shows that there is a difference between ethylethane sulfonate and methylmethane sulfonate, whether we take all of the nucleotides or whether we consider one particular nucleotide, as we probably do for reverse mutations.

The question is, how can one explain this? I will first give a theoretical explanation, and then an experimental result. The theory is that when you attach the alkyl group, either the ethyl or the methyl, at the 7-position, you get the positive charge in the ring, but the more electrons this alkyl group can provide to the ring the smaller the positive charge will be.

It is known from chemistry that the methyl group is the better electron donor than the ethyl group. This should make the ring less positive, and therefore the likelihood that a group breaks loose is less.

Actually, Zamenhof, in his first paper, could observe the methylation of these bases, but he had difficulty in finding any ethylation. I think that this is automatically explained because the methyl groups are more stable than the ethyl groups.

The experimental part is that we treated deoxyguanosine with diethyl sulfate and with dimethyl sulfate, and then determined in the fraction collector the relative amounts of guanine, methyl guanine, and methyl deoxyguanosine and similarly for ethylation.

I don't want to go into the details of the calculation, but we found that the probability of decay of a methylated guanine is of the order of five times less frequent that the probability for an ethylated guanine; this is in agreement with both the theoretical expectations and the observations about mutagenicity.

Auerbach: There is one group of chemical mutagens which I should mention, although I don't think they have been tried on phages. Those are mutagens which perhaps chemically have the best claim to being called radiomimetic. This is a very loose term which is used often for mutagenic chemicals.

There is one group of mutagens to which, possibly, this term applies at the chemical level—the peroxides. Hydrogen peroxide is a weak mutagen, but it can be definitely mutagenic, at least under conditions where it is not destroyed at once. It is not mutagenic for Drosophila. Apparently it is destroyed at once by catalase. It is weakly mutagenic for bacteria. But various organic peroxides have been found to be good mutagens in Neurospora as well as in Drosophila.

Now, how does this link up with radiation mutagenesis? There is a lot of discussion as to how far radiation produces its effect indirectly via water radicals, including their combination to hydrogen peroxide. I think that the extent to which this happens is still very doubtful. The pendulum seems to swing back again toward attributing more importance to the direct effect on the target molecules. But there certainly is one case, well authenticated, where radiation mutagenesis acts

via peroxide. The Texas school of workers found that mutations can be produced in bacteria by irradiation of the medium on which they are subsequently plated (69). When they analyzed this mutagenic effect of irradiated medium, they found that the responsible mutagens were amino acid peroxides.

Thus, the group of peroxides forms a link with radiation mutagenesis. Curiously enough, it seems that formaldehyde, at least in one type of experiment, should be included in this group. Formaldehyde is a very curious mutagen. In Drosophila, where Rapoport first discovered its mutagenic action (58), it can be applied in two different ways, and I think it acts quite differently when applied in one or the other way.

The first way, which was discovered by Rapoport, was to mix the formaldehyde with the food of growing larvae. I shall come back to this in a moment. The second method, which was introduced much later (3), was to inject an aqueous solution of formaldehyde into adult flies. This is similar to the way in which it is applied to Neurospora (18), where spores are treated in an aqueous solution. Under these conditions, formaldehyde seems to produce mutations by forming peroxides with metabolically produced hydrogen peroxide. The evidence is this: When Neurospora conidia are treated with hydrogen peroxide, there is a very small mutagenic effect. When they are treated with formaldehyde, there is again a small effect. When they are treated with a mixture of the two, there is a very strong effect. Sobels (65) in Holland found further evidence for this interpretation. He treated Drosophila with the organic peroxide that is formed through the reaction of formaldehyde and hydrogen peroxide, and it produced mutations.

When formaldehyde is mixed with the food of the Drosophila larvae, it probably acts in a completely different way. It has a very specific effect then. It acts only on growing spermatocytes. It does not act on females at all, nor even on adult males. It acts only during one very particular stage, occurring only in the growing larvae, where the developing spermatocyte increases tremendously in growth, and where, presumably, very many synthetic processes are going on. In fact, Alderson (1) showed quite recently that when the larvae are reared on a chemically defined sterile medium, formaldehyde acts only in the presence of adenosine riboside. Certainly this indicates that its mutagenic effect is somehow connected with the synthetic processes going on during the sensitive stage.

I think that covers the main groups of mutagens. There is still

manganese chloride. This, I think, is the most curious mutagen of all. So far, it has produced mutations only in bacteria and only in certain strains of bacteria, although it has been tested on other microorganisms. Did it act on Salmonella, Dr. Demerec?

Demerec: Yes, it does.

Auerbach: But much more on *E. coli*?

Demerec: Yes.

Auerbach: And even in *E. coli,* more on certain strains than on others. In Penicillium, it does the opposite. It is an antimutagen to nitrogen mustard. I think, in this case, the explanation which Dr. Demerec gave long ago is correct—that it acts in some rather unspecific ways by disturbing the metabolism of the bacterium and probably upsetting defense mechanisms which preserve the integrity of gene reproduction.

Neel: I wonder if Dr. Demerec would summarize the work with manganese chloride now?

Auerbach: May I say something? Dr. Demerec told me a very interesting story about aminopurine yesterday. I was just wondering whether he could repeat this now.

Demerec: The work with manganous chloride was published in an extensive paper in 1951 (15). It was done in the early days of our research with bacteria, when we were developing techniques for studying induced mutagenesis in *Escherichia coli* and had begun testing chemicals to see which ones were mutagenic. In our survey, which was done with Joe Bertani (14), we included 31 chemicals representing some widely separated groups. Nineteen were found to be mutagens. Among these was manganous chloride, with which studies were continued because it showed several unusual properties. In the first place, it was a very potent mutagen, inducing mutations under conditions of treatment that produced very little or no killing. Moreover, its mutagenicity could be modified by certain pretreatment and posttreatment of the bacteria. We found that the mutagenic effect increased linearly with concentration, up to $2.5 \times 10^{-4}M$, and with time treatment up to 30 minutes, after which further increases in either concentration or time were ineffective. Washing of the bacteria before treatment, in water or in hypotonic solutions of NaCl, KCl, $CaCl_2$, sucrose or dextrose decreased the frequency of induced mutation, whereas washing in hypertonic solutions of these compounds increased it. Washing in similar concentrations of various other salts resulted in lower mutation frequencies than washing in NaCl or KCl, in the following descending order: $CaCl_2$, $BeFl_2$, UO_2Cl_2, $CdCl_2$, $CrCl_3$, $CoCl_2$,

$MgCl_2$, and $ZnCl_2$. Tests involving 18 Na salts indicated that the effect of washing depends on the concentration of Na ions in the washing solution rather than on its pH. The mutagenicity of $MnCl_2$ was tested under several other conditions. As was mentioned by Dr. Auerbach, the explanation given for the mode of action of $MnCl_2$ is that in some unspecific fashion it affects the metabolic activity of bacterial cells in such a way as to make the gene systems more unstable than they otherwise are.

Neel: Is there any other chemical whose effects are so modified by your pre- and posttreatment as this one?

Demerec: From among the large number of chemicals tested, only $MnCl_2$ and a few related compounds were found to be highly mutagenic and reactive to pretreatment (16). They were: manganous sulphate, nitrate and acetate, and ferrous chloride and sulfate. They did not include manganous lactate, ferric chloride or sulfate, or several stannous, cerous, cobaltous, and nickelous salts.

Goldstein: Were experiments ever done with posttreatment with a chelating agent like EDTA?

Demerec: No.

Lederberg: On the mechanism of action of manganous ion, I think you yourself suggested the possibility, perhaps even the probability, that this is functioning in an intermediate. Peroxide plus ferrous iron is a very active oxidizing agent, and I would be surprised if this weren't true to some extent with manganese as well. Has this been clarified at all?

Demerec: No. You see, this work was done before it was known that peroxides were potent mutagens and that they may be responsible for the mutagenic effect of ultraviolet radiation.

Auerbach: But it is such a specific mutagen.

Lederberg: That may be a matter of being in the right place in the cell. Even posttreatment effects may have something to do with having localized it.

Auerbach: No, I mean in the sense that only certain strains react with it, even in *E. coli.*

Lederberg: Presumably, it has to complex with something in the cell or it won't stay there.

Auerbach: But wouldn't you expect that this might happen in every cell, not only in very special ones?

Lederberg: I'm sure that if you fixed manganous iron or ferrous iron with hydrogen peroxide, you would get tremendous lethal effects.

Zamenhof: No, not manganous. Ferrous iron reacts strongly (86, 91) but manganous does not.

Lederberg: Doesn't the manganous do that? You know that from experience.

Zamenhof: Yes. It has an entirely different redox potential. Ferrous iron autooxydizes in the air but manganous does not.

Novick: Manganese is a free radical agent.

Lederberg: In reacting with oxygen?

Novick: No. All kinds of free radicals.

Lederberg: Of course, it does pick up an unpaired electron or it could donate an electron to form a pair of radicals. I suppose one question would be the relative effectiveness in aerobic and anaerobic conditions.

Demerec: Anaerobic is lower in effect than aerobic.

Lederberg: Is it a very pronounced difference?

Demerec: Quite pronounced, yes.

Novick: What about the correlation of different kinds of mutations? Have you tried more than one?

Demerec: No. Only one kind of mutation was studied in detail, namely, reversions from streptomycin dependence to nondependence. At the time this work was done we assumed that we were observing back mutations, but later work by Hashimoto (34) revealed that these reversions are due to forward mutations in a suppressor locus, which is closely linked to the region controlling streptomycin resistance. However, we have evidence that $MnCl_2$ is a potent mutagen for the genes controlling resistance to phage T1, lactose fermentation and five steps in the synthesis of essential nutrients. I wish to add that Roberts and Aldous (61) studied manganese metabolism, using our strain of *E. coli,* and concluded that the manganese is adsorbed on proteins or nucleic acid of the cell in a loosely bound complex, and that the quantity adsorbed is determined mainly by the concentration of manganese and other competing ions, and by the number of available adsorption sites.

Auerbach: In Penicillium, Sermonti (64) used manganese chloride, and it had a very unexpected effect. Not only did it not produce mutations or crossovers, but it suppressed the mutations which had been induced by nitrogen mustard. I think that it acts in a very indirect way.

Demerec: You have asked me to say a few words about our current work on mutations induced by 2-aminopurine and several other agents. This work is being done with strain LT_2 of *Salmonella typhimurium.* We have a collection of about 4000 mutants, each of which originated independently by forward mutation from the wild type. We find, as has been reported in phages, two classes—those that have the capacity

to revert to the wild type, and those that cannot revert. Mutants of the first class are demonstrably the result of mutation at a single site of a gene locus, whereas the nonreverting mutants are due to mutation affecting two or more adjacent sites. These so-called multisite mutants, since they show properties characteristic of the deletion mutants found in higher organisms, are assumed to originate by deletion of a small segment of the gene string, and I shall refer to them as deletions. As a rule, the frequency of deletions is low, about 4 per cent, as compared with the frequency of single-site mutations. The only exception detected so far is at the *cysC* (cystine-controlling) region, where close to 40 per cent of the spontaneously occurring mutations are deletions. All the deletions above 4 per cent found in the *cysC* region form a uniform group. Detailed studies of 44 of them showed that they are very similar to one another with regard to the positions of the two ends, one being in the left third of the *cysC* region and the other either at the right end of *cysC* or beyond that end of the locus. The available evidence makes it seem probable that the sectors within which the two ends of these deletions are located are duplications, and that the deletions originated during replication of the gene string by the formation of a tight loop and subsequent exclusion of the portion within the loop from the newly formed string. The other deletions are variable in length and cover different parts of the *cysC* region. Since the additional deletions are longer than the others, they are called "long deletions," whereas the others are classified as "short" even though some are of considerable length. By dividing the deletions into two classes I do not wish to imply that their modes of origin are different. It seems likely that all deletions originate by the subtraction of nucleotide pairs because of "skips" during replication, due either to sliding between adjacent nucleotides of a pair or to the formation of loops. In the *cysC* region a duplication of considerable length would be responsible for frequent synapsis of duplicated segments and the formation of loops. Work on this problem is still in progress. The results available through January 1961 are given in Table 2. Additional experiments are now being made with sodium nitrite and UV. The data show clearly that 2-aminopurine induces only single-site mutants, whereas all the other mutagens induced long deletions as well. It appears that, of the five mutagens tested, sodium nitrite is the most potent inducer of deletions. Short deletions are so rare that the mutagenic differences shown in the table are not significant.

Freese: What occurs more frequently with X-rays, long or short deletions?

TABLE 2

Numbers of single-site mutations, long deletions, and short deletions among *cysC* mutants of different origin. Factor by which number of mutants is larger in treated series than in control series; 2AP, x125; UV, x10; X-rays, x20; neutrons, x11; $NaNO_2$, x12.

Origin	Total	Single-site	Long dl	Short dl
Spontaneous	52	34	16	2
2-Aminopurine	54	54	0	0
UV	21	13	8	0
X-rays	51	31	17	2+1?
Neutrons	37	29	8	0
Sodium nitrite	17	7	7	3

Demerec: Long, as I remember. We have three short deletions in X-rays, and we have three short deletions in total, with spontaneous and ultraviolet. Now, this would not be very significant, but we sent histidine-induced mutants to Phil Hartman at Johns Hopkins. He is interested in the analysis of histidine loci, and has analyzed a considerable number of histidines. He tells me that he finds short deletions present more frequently among x-ray induced mutants than among spontaneous.

Lederberg: You mentioned the figure, 4 per cent. Is that the fraction of short deletions among all deletions?

Demerec: No, that is the fraction of the deletions among all mutations found in 23 gene loci, not including *cysC*.

Benzer: Is there some other feature that distinguishes the UV and X-ray ones from the spontaneous?

Demerec: We cannot say.

Benzer: Could they be spontaneous ones which have somehow been enriched?

Demerec: That seems very unlikely, because the same procedure—the penicillin technique—was used to isolate auxotrophs in all the experiments. In the UV experiments, as the table shows, mutants were about ten times more frequent in the treated series than in the untreated controls.

Goodgal: The deletions you were talking about, the long deletions, were they selected in separate experiments?

Demerec: Oh, no! They were found during an analysis of *cysC* auxotrophs isolated in different experiments.

Goodgal: But do you mean to say that they are all independent?

Demerec: Yes, they are independent. We took the necessary precautions to insure that all the mutants mentioned in Table 2 originated independently of one another.

Auerbach: The duplication creates a special situation. It is a means for producing deletions.

Demerec: Yes. I was just talking about the mechanism for induction of mutants. I think that it is a loop formation, even in short deletions.

Lederberg: But if you have a duplication that is functional, there is a bias in favor of deletions, because the point inactivation would have no phenotypic effect.

Auerbach: Except the ones in the region between.

Demerec: If the presumed duplication consisted of genetically functional segments, a single-site mutation occurring within a duplicated region could not be detected, and the observed number of single-site mutants would be smaller than the actual number of mutations with the result that there would be a bias in favor of deletions. If the duplicated segment is genetically nonfunctional, however (a "nonsense" region), and I am inclined to believe that it is, no such bias would exist. In any case, even the existence of a bias would not affect the conclusion that can be reached from the data.

Freese: Has anybody looked at the induction of mutations after P^{32} incorporation?

Demerec: No. It is a quite laborious process to get those.

Freese: There might be a difference between phages and bacteria; all those phages in which the chromosomes are broken are probably lost, while in bacteria such broken chromosomes may possibly be saved by some healing process.

Zamenhof: One of the chemical mechanisms where short "deletion" could occur is a reaction in which the sugar is destroyed. The agents which will destroy the sugar should produce short "deletions." We think that this may happen when you get "deletions" by heating in the dry state. The reaction which occurs there is not always hydrolysis, where you only split off the base, but it may be pyrolysis (84); there may be a dehydration and the sugar may be lost.

Demerec: This means that the sugar is lost and all the bases which are attached to that part are lost.

Zamenhof: If the sugar is lost, the nucleotide will never reproduce; while in certain cases, heat could produce just the loss of purine, in other cases, even in the case of pyrimidine nucleotide, if the sugar is destroyed, the corresponding nucleotide would be completely lost.

Demerec: Could you lose several nucleotides?

Zamenhof: You probably could.

Demerec: Because that is where the short deletions come in.

Auerbach: But the long deletions come in, too.

Demerec: No, the long ones take about 75 of the identified sites.

Auerbach: There was a somewhat similar story by Yanofsky (82) in a strain of *E. coli* where he almost always got deletions embracing two loci in a particular chromosome region, and this property was transmitted in crosses. Yanofsky does not, I believe, mention this possibility, but could this not also be a region with a duplication and the tendency to form a loop?

Atwood: What were the criteria for deciding that part of the included region in the deletions is inert?

Demerec: The long deletions cover all the sites that have been identified in the right end of the *cysC* locus, and it seems probable that the deletions terminate in the region beyond the locus. I have a feeling, but unfortunately no evidence, that nonsense (heterochromatic) regions frequently intervene between loci that do not belong to a sequence. Meager support for this belief has been obtained from our studies of proline mutants (51). If the region to the right of *cysC* is heterochromatic, and if a portion of it or of any other heterochromatic region is duplicated in the left part of *cysC*, one could expect synapsis between the duplicate sections to be just as frequent as if the duplicated segment were functional.

Atwood: If you went over your shorter deletions, you might find one or more of them that covered only one of the presumed duplicated regions, and if that is the reason for its apparent inertness, then, using that stock with one part deleted, you would find point mutations in the remaining part. That is one way of getting it. Those could be surveyed to see if they get new point mutations. What I mean is you could survey them to see whether there are any of them which, when treated again, now yield a new class of point mutations not yielded by the original stock, because the redundancy has been taken out by the deletion.

Benzer: But these are mutants, and you can't see any new point mutations.

Demerec: There is no way of selecting them.

Atwood: Oh, you have already selected them? You can't do it by—

Benzer: The double mutants will look just the same as the single.

Atwood: You don't delete the same way?

Benzer: You have to test each one.

Atwood: It is possible, but it would require doing it without screening, which is then almost impossible.

Auerbach: In fact, if that is the explanation, short deletions in one of the redundant segments would not even be picked up, because it would still be—

Atwood: Well, it has the part that overlaps, the nonredundant part, then.

Benzer: I'm afraid you would have to stretch the duplication idea to having duplications within duplications within duplications, because I can give you a set of big deletions all the same, a smaller set all the same included in the first one, and a third one included in the second.

Demerec: Why not, particularly if the duplicated segments are heterochromatic? There is good evidence in Drosophila work that heterochromatic segments are scattered throughout the chromosomes (33). One suggestion is that it may be an effect on polysaccharide, and the other is just an accidental formation of the loop without any duplication, and so on. You have uniformity there; they are all just the same, and a large number of them indicate or suggest duplication.

Goldstein: Some of us don't understand the connection between the data you presented and the logic that leads you to talk about duplication. Could you explain that?

Demerec: The evidence in support of duplication has not been mentioned, and I am glad that you have raised the question because we have studied it in some detail. The results are described in the last Annual Report of the Department of Genetics (16), and I shall summarize them briefly. All the long deletions of *cysC* are similar in length. One end of each falls within a section occupying the left third of the locus, and the other end is either at the extreme right of the locus or beyond its right-hand boundary. It is possible to analyze the left-hand section because genetic markers are available to either side of it. The frequency of recombination between these markers indicates that the section is of considerable length, comprising almost one-quarter of the locus. Although more than a hundred single-site mutations have been identified in *cysC*, none of them is located within that section—which thus appears to be genetically silent, as would be expected of a duplication whether it consisted of genetically functional or of genetically inert material.

Stern: If this is a duplication, I would say it is at a selective disadvantage from the point of view of producing deletions constantly.

Therefore, how did it get established in evolution? It would be established if it also has selective advantage as being a duplication at the same time, which would overpower the disadvantage of its instability. Is that correct reasoning?

Auerbach: You wouldn't find mutations. Also, the silent mutants in this region would be protected. That may outbalance this disadvantage.

Lederberg: Could I raise this bugaboo—why there should be more than four mutation rates and just what chemical basis there might be for higher order differences in different parts of the DNA? Does anybody have some ideas on this?

Glass: I have a somewhat speculative question that I would like to ask in regard to the peroxide (and perhaps it relates as well to the other radiomimetic chemical mutagens).

We now know that in all organisms that carry on photosynthesis with chlorophyll, where water is split, a big step is the formation of hydroxyl radicals which results in the liberation of molecular oxygen. The peroxide phase that results from the combination of the hydroxyl radicals is very brief and the concentration of peroxide is never permitted to rise in the cytoplasm, because of the deleterious effects that it would produce in general.

The questions which this brings to my mind relate on a comparative basis to the possible differential action of such mutagens in microorganisms, in multicellular animals, and in green plants. The effects with peroxide were originally discovered in microorganisms, and my first question would be, does this mean that in the microorganisms there is an abundance—even in the photosynthetic bacteria which do not split water—an abundance of catalase or peroxidase or at least peroxide-destroying enzymes, or is there a lack of these that would permit the action of the mutagens much more strongly than in green plants?

The second question that arises from this consideration is whether peroxide-destroying enzymes are limited to the cytoplasm, and whether, therefore, the production of hydroxyl radicals or of peroxides in the nucleus would be able to act on the genetic material, whereas if peroxide were produced in the cytoplasm it would normally be neutralized before it could ever have a chance to act. I would ask whether anyone knows whether there is good evidence of the existence in green plants of a real peroxide effect when peroxide is applied externally? Can it get through the cytoplasm to the genetic material in the nucleus and

produce mutations, and if so, is this true only of organic peroxides, or does it apply also to hydrogen peroxide?

Auerbach: As far as I know, this has not been tested. To my knowledge, the only organisms in which peroxides have been used are Neurospora and Drosophila.

Glass: And in Drosophila?

Auerbach: They are effective.

Glass: Hydrogen peroxide, that is?

Novick: Not in *E. coli.*

Auerbach: Not hydrogen peroxide; this is destroyed almost immediately by catalase.

Glass: These are organic peroxides?

Auerbach: Yes.

Glass: They were added to the food?

Auerbach: No, injected.

Benzer: Hydrogen peroxide does act to induce lysogenic bacteria.

Auerbach: It is very weakly mutagenic by itself, but it is very much reinforced by formaldehyde, or the other way around.

Novick: Be careful about generalizing, because there are all kinds of bacteria. So-called lactic acid bacteria lack it completely.

Benzer: And people too. There is the story about the Japanese dentist who swabbed his patient with peroxide, and it didn't foam. It turned out to be due to a mutation.

Neel: We have a paper from that Japanese dentist in the *Journal of Clinical Investigation* of a few months ago (73). There are now some 17 families reported in which there have occurred individuals who apparently are completely lacking in the enzyme catalase. Even more interesting is that the carriers of the responsible gene, which behaves as a recessive, have approximately half normal catalase levels, and, if you take the usual Hardy-Weinberg assumptions, which are not good assumptions at all in human populations but give you a first approximation of what you want, there must be 300,000 or so Japanese with half normal catalase values, and no obvious ill effects.

As a matter of fact, we suspect that complete absence of catalase may sometimes be compatible with normal health. Some of the cases of acatalasemia have been discovered quite by accident. The first affected individuals were detected because of an ulcerating gingivitis, which was really life-threatening, but now it begins to look as if some affected individuals may be quite normal phenotypically and are discovered accidentally at the time of unrelated surgery.

Bearn: We have looked for acatalasemia carriers. So far we have

examined 300 Caucasians from New York City without finding any cases. We have also examined hemolysates from 63 Japanese in Seattle without luck.

Glass: Will these familial enzymes act on organic varieties of peroxide, do you know?

Auerbach: If it is true that formaldehyde when used in solution acts by forming the addition compound with hydrogen peroxide, and that this addition compound is the actual mutagen, then the enzymatic constitution of the organism becomes important. On the theory that catalase poisoning would enhance the mutagenic effect of injected formaldehyde in Drosophila, Sobels (66) pretreated flies with potassium cyanide. He did increase mutation frequency, and even obtained some mutations in females, which ordinarily are quite refractory to the treatment with formaldehyde.

Freese: I would like to pose a problem here. Peroxides may act either as such or as radicals which are derived from the peroxide. Since organic peroxides are active mutagens, while hydrogen peroxide itself does not seem to be effective, the possibility of radical action seems more likely.

Lederberg: I certainly agree with Freese on that point, but we did find that peroxide was quite effective in inducing the segregation of diploid heterozygous bacteria, which would indicate that it is at least inducing lethal lesions (44). We haven't ourselves studied it for its mutagenic effect, but it may cause widespread damage.

Auerbach: Hydrogen peroxide?

Lederberg: Yes. Whether it is hydrogen peroxide by the time it reaches the DNA is an entirely different question. It may be entirely a matter of dose and concentration and what it can react with as a carrier on the way in. Most of the bacteria that have been used in genetic research do have catalase, and I think this is something of a problem.

In following up what Dr. Glass had to say, I think it should be remembered that quite a number of compounds do have catalase activity to varying degrees. Even inorganic iron does, to a very substantial extent, and heme and hemoglobin do have a measurable catalase activity. It may run throughout difficult quantitative questions in trying to decide what the role of catalase is in protecting mutagenic material.

Auerbach: In the Texas experiments by Stone, Wyss, and Haas (69), it was not hydrogen peroxide which was effective, but organic peroxides.

I come now to another subject altogether. This is one which I have

found particularly fascinating, right from the beginning of my work with chemical mutagens. The possibility was raised already whether chemical mutagens may act more specifically than radiation, whether certain loci respond to certain mutagens better than to others.

There is no doubt that this is true in an operational sense. There have been many instances now where the pattern of response to a variety of mutagens differs between loci. What I want to discuss and also want to study myself is at what level of the mutagenic process this specificity happens.

Dr. Zamenhof has stressed again and again the fact that mutation is a complex process, a process with many steps, and I want to discuss each of them separately to show that what is observed as mutagen specificity may occur at any one of these steps.

I want to say one thing beforehand, for those who are interested only in the decoding. In using chemical mutagens as a means for decoding the DNA there is, of course, no interest in mutagen specificity at any level except that of the DNA. All other specificities are just obstacles, and so the kind of work which I am going to discuss now, the kind of approach, will have only negative value because it removes red herrings. But if one is interested in the whole process of mutagenesis in the cell, at the cellular level, I find that chemical mutagens and their specific effects may be very useful tools for unraveling what happens at these other steps.

If we have a nonmutated gene in a nonmutated cell, the mutagen has to reach the gene. It has to penetrate to the gene and also, presumably, will interact with the cytoplasm. I think in higher organisms one should not forget that it also has to interact in some way with the protein with which the gene is associated.

After this comes a step which leads to—I forget what Witkin calls it—the premutated gene. This state certainly occurs after UV and possibly also after other mutagens. In this state the gene still has, so to speak, a choice. It may recover and go back to the original condition or it may go on to the actual mutated state. After this, we have a mutated gene in a nonmutated cell; this mutated gene now has to change the biochemical pattern of the cell, which it has to do in the face of competition from other cells in the same environment, and it has to overcome obstacles there which as I shall show with some examples, may lead to specificity. Finally, then, we end up with the mutated gene in the mutated cell.

I want to illustrate this by a few examples. Mutagen specificities in macroorganisms are complicated to analyze because it is so difficult to

distinguish between specificities that occur at the level of the gene and specificities that refer to chromosomes or chromosome regions. What appears to be gene specificity may in reality be specificity of a certain chromosome region. The centromere regions or heterochromatic regions may be more likely to react to a certain mutagen. In addition, with most materials we don't know whether we are dealing with gene mutations, with deletions, with position effects, or with rearrangements.

This does not take away any of the potential practical value of this work. Quite a lot of it has been done by Swedish workers on barley (79). They find mutagen specificities in regard to chlorophyll mutations. The "spectra" of albino, yellow, light-green, etc., mutations are different for different mutagens. But it seems to me that there are fairly strong indications that the more drastic mutations, especially the albino mutations, tend to be connected with chromosome rearrangements or deletions, for they are mainly produced by the more drastic agents like neutrons or X-rays. From the point of view of gene or allele specificity it would therefore not be fruitful to enter into a discussion of this work. The same applies at the moment to Drosophila.

Russell: If you're looking for examples where the spectrum of induced mutation rates differs in different gametogenic stages, you could cite the mouse.

Auerbach: You mean the specificity is different?

Russell: The ratio of rates of different loci is quite different for spermatozoa and spermatogonia.

Auerbach: With X-rays?

Russell: Yes, and it is probably different with the oocytes, too, although we don't have enough data to be sure.

Auerbach: I always find it difficult to imagine which ways X-rays can act specifically at this level.

Russell: In the mouse, we have suggested an explanation, namely, that the specific locus mutations recovered from irradiated spermatozoa include things other than point mutations, perhaps even fairly large deficiencies, whereas the mutations induced in spermatogonia may be almost entirely point mutations, or if they involve deficiencies, deficiencies so small that we are unable to detect them (62).

Auerbach: That would mean that the point mutations are more frequent in spermatogonia?

Russell: The relative rates for point mutations at different loci may be the same in spermatogonia and spermatozoa, but superimposed upon the point mutation rate for spermatozoa, there may also be chromosomal types.

Auerbach: But that should make it more uniform in spermatozoa.

Russell: The absolute rate at all loci is much more uniform.

Auerbach: Oh, yes, that's what I meant.

Neel: Bill, would you expect that following spermatozoa irradiation, the apparent "specific locus phenotypes" would tend to be accompanied by other abnormalities if deletions are a significant part of the picture?

Russell: Yes.

Neel: And do you see accompanying abnormalities?

Russell: Most of the specific locus mutations, even those induced in spermatogonia, are completely lethal homozygous, so we would have to look for this mainly in heterozygotes. I would say, in general, that the ones induced in spermatozoa are not as viable as those induced in spermatogonia. Some of them are associated with translocations and some are clearly deficiencies, because we have two markers close together which go out at the same time. I think there is a greater amount of size reduction in the mutant animals themselves for those induced in spermatozoa, but this hasn't really been put on a quantitative basis.

Auerbach: Then I come to microorganisms, where there is abundant evidence for mutagen specificity at the observational level. I think what I have to say will sound rather naive, but I am really interested in discussing for a few examples at which stage of mutagenesis the observed specificity is likely to arise.

Glass: Before you begin with the microorganisms, would you say something about the work of the Fahmys (22)?

Auerbach: You will find a criticism of their latest claims in the present number of *Genetics* (5). I wrote it together with a statistician, and the conclusion we arrived at was that their experiments do not disprove the possibility of mutagen specificity in Drosophila (21), but that they do not prove it either.

However, the Fahmys have meanwhile started on what to me seems a much more promising line. Their work so far had been concerned with testing whether certain visible mutations occur more frequently after one type of treatment than after another. Now these visible mutations are "forward" mutations and presumably occur at many sites within a locus, so one hardly would expect much mutagen specificity among them. But now the Fahmys have started to analyze one particular locus, the "rudimentary" locus, into what they claim are different cistrons. They have published a very short note (22) in which they say that these cistrons may differ in their response to mutagens. If this can be substantiated it would be more in line with

what is known from microorganisms and would certainly be very interesting.

To return then to mutagen specificity in microorganisms. I am not going to deal with forward mutations—I did that yesterday. Heslot's data are the only relevant ones I could find on this point.

At the same conference in Gatersleben where Heslot (35) presented these data, Westergaard (78) gave data on forward mutations in Penicillium, and these have been published in the same Symposium volume. Westergaard did not find any specificity and he did, in fact, not expect any. He classified the mutants phenotypically, by their requirements, and one would hardly expect this to result in specificity.

I shall confine my discussion to reverse mutations from auxotrophy to prototrophy, and I want to describe the technique of these experiments because this happens to be very important. Let us take an example. Let us assume we screen for tryptophan reversions in either Neurospora or bacteria. First, one grows the organisms in or on a certain medium, then one makes a suspension, which is then distributed into two tubes. One tube is treated, the other is the control. After treatment one plates, which is a procedure taken too much for granted. Let us look at it a bit more closely. There are two sets of plates, one for determining viability, the other for determining mutation frequency. Both of these are important for the calculation of mutation frequencies, for in bacteria always, and in Neurospora usually, mutation frequencies are calculated per survivors. Even if they are calculated per numbers plated, this number is derived from the colonies formed on the viability plates, not from direct counts of cells treated. It is important to keep in mind that the two sets of plates differ, and they differ in two respects. In the particular example chosen, the viability plates have tryptophan, the mutation plates have not. The other difference is that the viability plates are plated at low density, say one hundred to a few hundred cells per plate, while the mutation plates are plated at very high densities, say 10^7 or 10^8 per plate. This is something I want you to keep in mind.

Now to come to causes, possible causes of specificity. The first one occurs at the stage where the mutagen has to penetrate, has to make its way through the cytoplasm and through the gene-associated protein in order to produce a reaction with the DNA. This may already produce what appears as specificity. Of course, one tries to compare different mutations on an isogenic background; that is to say, one preferably would compare mutations which have arisen spontaneously in the same strain. But one should not overlook the possibility that

the mutation itself, whose response one is testing, may alter the biochemical pattern of the cell.

I might mention the heminless bacteria which, when they are grown without hemin, have no catalase. Naturally, they will react in a different way to radiation. One way to overcome this, and the way which is usually used, is to have doubly marked strains and compare the frequency of mutations of two loci on the same background. Then one gets a typical pattern, which would look like this:

	Mutation frequency	
	Mutagen A	B
gene a	high	low
gene b	low	high

When such a pattern has been obtained work on it usually stops, and only two interpretations are considered. The one is that this is specificity of the reaction between gene and mutagen; the other, that the mutagen selects spontaneously arisen mutants. The latter can be ruled out by reconstruction experiments, and this is often done. But all other possibilities are usually neglected.

There is, for instance, a very obvious one. It is the fact that when you use a double strain, the medium for testing mutations is not the same for the two mutants. Let us say we work with a tryptophanless leucineless strain. If one tests for tryptophan reversions one plates on leucine; if one tests for leucine reversions one plates on tryptophan. The influence of this difference in plating medium has, to my knowledge, never been tested. A collaborator of mine, Dr. Clarke, is just testing it in fission yeast, *Saccharomyces pombe*. He has already observed one striking effect of the plating medium. He scores for reverse mutations at two loci, the adenine-1 locus, and a methionine locus. He found that methionine in the medium suppresses mutation at the adenine locus, so that in the double strain, when he plates on methionine for adenine reversions, these are very much reduced in number. If this kind of response to the plating medium should be influenced by the previous mutagenic treatment—and I see no reason why this should not be so, especially after chemical treatment—then one would obtain a mutagen specificity which occurs at a very late stage in mutagenesis, when the premutation has to become stabilized or even later when the mutated cell has to make a colony.

I have been wondering whether any of the cases of mutagen stability can be due to such an effect of the plating medium. Take for instance Glover's (30) interesting data on *E. coli*. He treated a tryptophanless strain with a variety of mutagens and scored reversions. In a certain experiment, he obtained 249 reversions with UV, 55 with X-rays, and none with diepoxybutane. So it seemed that this tryptophan allele was specifically mutagen stable to diepoxybutane. But when he incorporated an arginine requirement in the same strain, the tryptophan locus responded to the chemical. Witkin has recently reported a very similar case. I feel that in these cases one should at least see whether the plating medium is responsible, whether, for instance, in Glover's case the apparent mutagen stability of the tryptophan locus in the wild-type strain would disappear if one plated on arginine instead of minimal medium. I don't say it would, but it would be worth testing.

In addition, one must of course keep in mind that the whole biochemical pattern in a cell is altered by introduction of a second mutant gene, and that this alteration may affect various steps in mutagenesis of the first gene. Clarke found that in fission yeasts both plating medium and residual genotype influenced mutation frequency at the ad-1 locus, for while adenine reversions in the single adenineless strain were almost completely suppressed by methionine in the plating medium, suppression was less complete in the double strain with methionineless. It seems probable that the influence of the genotypic background, too, may depend to some extent on the mutagen used, and this is another possible explanation of the type of data Glover obtained. In this case, too, mutagen specificity would not be likely to reside in the reaction between mutagen and gene, for it is difficult to imagine that this reaction should be controlled by other genes in the same nucleus.

I would like to discuss a few more possible ways by which mutagen specificity may arise. One point that came out in the discussion this morning is that some and perhaps all alkylating agents go on acting for quite a long time after they have been washed out. This is so for diepoxybutane and TEM (tri-ethylene melamine), and Freese mentioned that it is so for ethylethane sulfonate. If there should be a difference in this delayed effect on two loci, then any initial difference in mutation frequency will be magnified the longer the material is kept between treatment and plating.

For instance, in the system used by Dr. Kølmark, which is an adenine plus inositol requiring strain of Neurospora, diepoxybutane acts preferentially on the adenine locus and very little on the inositol

locus. In one experiment on the delayed action of diepoxybutane, which Dr. Kølmark and I carried out together (42), we got the following results. When we plated at once, survival was about 80 per cent, mutation frequency per 10^6 survivors, and mutation frequency was 110 for the adenine locus and 5 for the inositol locus. When the suspension was kept for 4 hours at 30° between treatment and plating, survival remained sensibly the same, but mutation frequency at the adenine locus had increased to 830 per 10^6, while there had been no increase at the inositol locus. Thus, the simple expedient of keeping the treated suspensions for a few hours had intensified the difference between the two loci very much.

A more important source of mutagen specificity is one found recently by Witkin (81) in her work on UV induced mutations in *E. coli*. All her reverse mutations to prototrophy required stabilization of the premutated state, and this in turn required protein synthesis. She found now that this is not so for mutations from streptomycin sensitivity to streptomycin resistance, or from streptomycin dependence to independence. If, therefore, one would score reversions from auxotrophy to prototrophy and mutations from streptomycin sensitivity to resistance in the same strain, prevention of protein synthesis, for instance by chloramphenicol, would prevent mutations to prototrophy but not to streptomycin resistance. Operationally under these circumstances, there would be specificity of UV for mutations at the streptomycin locus.

Goodgal: I don't agree with that, because it may very well be that it is the postmutational step which is involved rather than the premutational step.

Auerbach: This is what I meant to say. I don't want to reinterpret or reassess Witkin's explanation. All I wanted to point out here was that you would get a marked specificity, which is not due to a specific reaction of the UV on the streptomycin locus.

Goldstein: Another difference here is not the difference between the tryptophan locus and the streptomycin locus, but the fact that the tryptophan locus is mutating in a backward direction from dependence to independence, and the streptomycin locus, from sensitivity to resistance, is mutating in a forward direction. And if the change from dependence to independence is a suppressor change, that is also moving in a forward direction. So you are comparing a backward mutation with a forward mutation, and this says nothing necessarily about the two loci involved. If one wants to ask a question about specificity differences between genes, then one cannot use a back mutation system,

because each mutant presumably—and I guess we'll hear from Dr. Benzer about this—will have its own particular pattern of back mutation, depending on the site where it is mutated.

Auerbach: There is another example from Witkin's work which may be relevant here. In her first paper, when she reported on the stabilization process, she also had tested leucine reversion and found it did not require protein in the plating medium. Leucine reversions and tryptophan reversions are both reverse mutations from auxotrophy to prototrophy, and if you had a leucineless strain and plated on minimal medium, UV would seem to act in a specific way on the leucine reversions and not the tryptophan reversions.

Goldstein: All I'm saying is that in going from auxotrophy to prototrophy you don't really know whether you're going backward or forward. One should use a system that is unequivocally forward for studying this type of comparison between genes.

Auerbach: What I said last was that the stabilization process might be influenced by the mutagenic treatment used. This seems to have been so in an experiment on tryptophan reversions in *E. coli* by Strauss (70). He treated with UV or with diepoxybutane. After treatment, he postincubated for one hour in liquid medium with or without a tryptophan analogue which prevented protein synthesis, and after this he plated on broth. Under these circumstances UV induced reversions were inhibited, while chemically induced ones were not. Strauss interprets this to mean that no protein synthesis is required for stabilization after treatment with diepoxybutane, but to me it seems equally likely that after the slowly acting chemical treatment one hour postincubation is not enough for the effect to show up, for when plating was done on minimal medium instead of on broth, chemically induced reversions, too, were inhibited by incubation with the tryptophan analogue. Whatever the interpretation, operationally this is again a case of apparent mutagen specificity, in which the seat of the specificity is not the reaction between mutagen and gene but a later step in mutagenesis, probably stabilization of a premutated state.

Once a mutation has been produced, the mutated gene has to establish itself and start a new biochemical pathway in the cell. After adenine reversions, the cell has to start making adenine, and after inositol reversions it has to start making inositol. To me it seems entirely conceivable and even very probable that the same chemical treatment which produces the mutations may interfere with the biochemical resources and potentialities of the cell in such a way that

priming for certain new processes is made easy and for others it is made difficult.

This idea was the starting point for experiments which Dr. Kølmark and I (4) did on the adenine plus inositol requiring strain of Neurospora. As I said already, the adenine locus responds strongly to diepoxybutane, the inositol locus hardly at all. Both respond well to UV, the inositol locus rather better than the adenine locus. In a cell that has been pretreated with diepoxybutane, UV produces many more adenine reversions than in a cell that has not been so pretreated. In some way, treatment with diepoxybutane sensitizes the adenine locus —but not the inositol locus—to a subsequent treatment with UV. This interaction need not occur at the stage when the mutated gene has to prime the cell for making adenine; it may occur earlier at the stabilization stage, and there are in fact reasons for thinking this more likely. Westergaard and his co-workers (50) found a similar interaction between formaldehyde and UV in the same strain. Again, formaldehyde sensitized the adenine locus, but not the inositol locus to mutation by UV, and this is particularly striking because formaldehyde itself is a very much weaker mutagen than diepoxybutane. In addition, these experiments showed another kind of interaction that has not been stressed by Westergaard, but that was quite pronounced. In a cell that had been pretreated with formaldehyde, UV not only produced more adenine reversions than in control cells, it also produced fewer inositol reversions. Whatever the final explanation may be, all these cases cannot be explained simply by assuming that there is a specific reaction of a mutagen with a gene.

Next the mutated cell has to form a mutant clone in competition with the nonmutant auxotrophic cells with which it is plated together. To guard against spurious specificity at this stage, one carries out reconstruction experiments, that is to say, one treats together the original auxotrophs and prototrophs of the kind that would have arisen by mutation. If the treatment, instead of producing mutations, selects for or against spontaneous mutants, it will cause an increase or decrease in the ratio of prototrophs to auxotrophs. Such reconstruction experiments are valid only if they are carried out under the same conditions as the mutation experiments, that is to say, with very small admixtures of prototrophs and very high plating densities. Even so, they do not really show the effect of competition between nonmutant cells and incipient mutants, for in several cases these latter have been shown to react differently from established ones. Ryan (63), for instance, has shown that incipient reversions at the histidine locus in *E.*

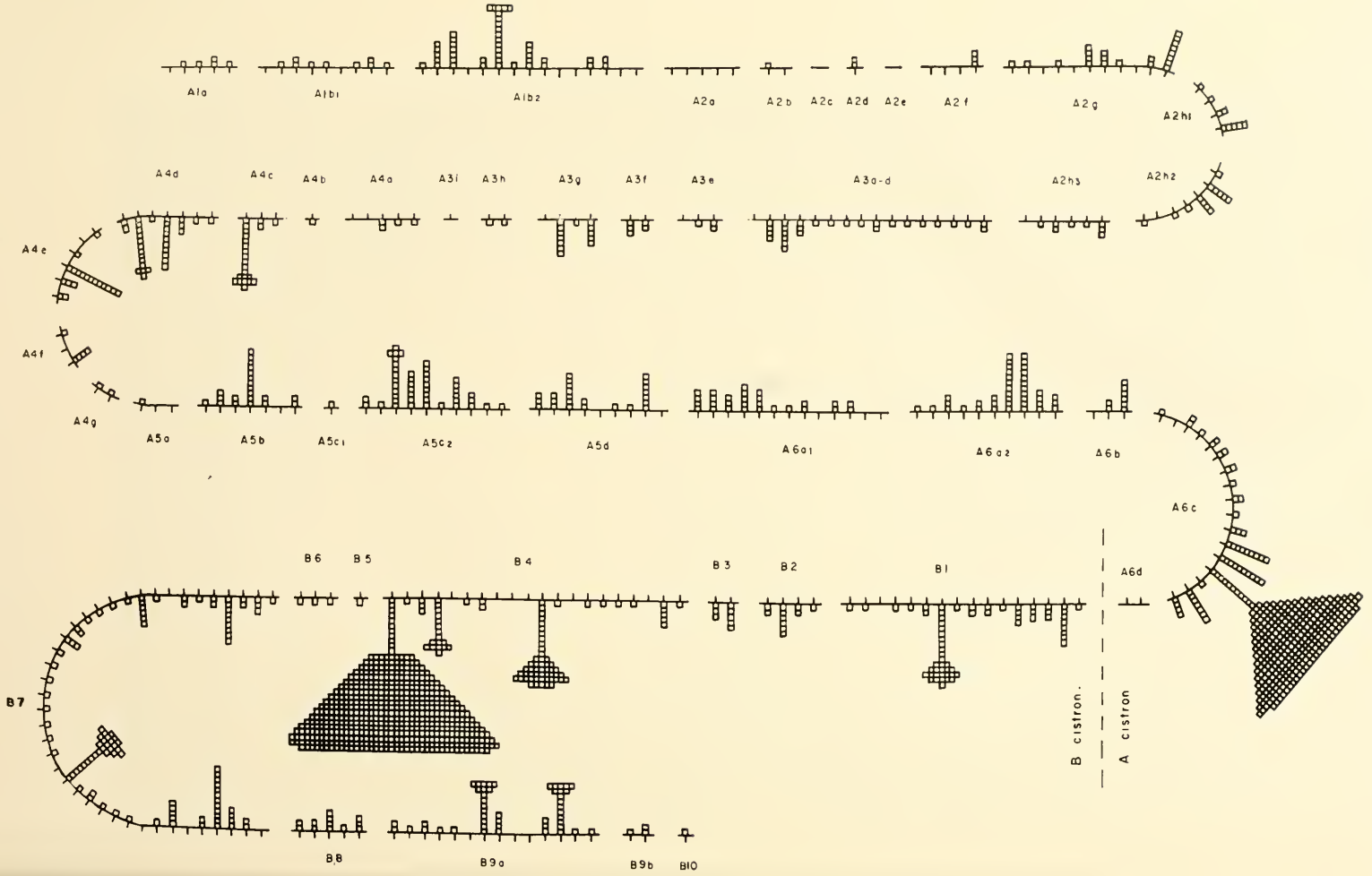
coli are much more sensitive to delay of mitosis by UV than already established prototrophs. Similarly, Kaudewitz (37) found in *E. coli* that immediately after the induction of lysine reversions by UV the mutant cells are exceedingly sensitive to very small changes in temperature.

Quite recently, Luzzati and his collaborators (49) reported an interesting case of this kind. They scored "gene conversions" or whatever you wish to call it in yeast cells that were heterozygous for two alleles of a gene causing a double requirement for adenine and histidine. In these strains, wild-type segregants arose through some kind of nonreciprocal recombination. The occurrence of such recombinants could be suppressed by histidine in the medium. Yet, in reconstruction experiments histidine did not discriminate against prototrophs.

In all these cases, an incipient mutation is affected by conditions that do not affect the established mutant. Any differences in the degree to which this happens when different mutagens are used and different mutations are scored would result in observable mutagen specificity.

Even in the ordinary reconstruction experiments, in which one tests for possible selection for or against established mutants, specificity may arise in response to plating conditions. You have to remember that plating density on the mutation plates is very high and one may obtain what is now usually called a Grigg effect, that is, a suppression of the prototrophs by the residual growth of the vast number of auxotrophs. Several years ago, Stevens and Mylroie (68) found interesting differences between strains of Neurospora in this respect. On plates with minimal medium, a large inoculum of leucineless spores inhibited the growth of a small admixture of prototrophs; an equally large inoculum of inositolless spores did not do so, presumably because there was less residual growth. In a doubly requiring leucineless inositolless strain, one might then expect suppression of inositol-revertants by residual growth of leucineless spores on minimal medium, but not suppression of leucine-revertants by residual growth of inositolless spores. Hollaender and his co-workers (92) found that after ultraviolet radiation there is a Grigg effect for mutations to purine independence, but not for mutations to streptomycin independence.

There remains one possible source of mutagen specificity that, to my mind, is the most difficult to detect. This is that the mutagenic effect of a treatment is measured by the ratio of mutants to survivors and that the numerator and denominator in this fraction are obtained under completely different conditions. I have already discussed this when I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A cistron

described the technique of these experiments. The difference between viability plates and mutation plates may lead to overestimates or underestimates of the living spores on the mutation plates, that is, of the spores that were actually tested for mutations. Hollaender and his collaborators (36) found that X-rayed bacteria survive better on a medium containing amino acids than on a medium not containing them. When amino acid revertants are scored, the viability plates contain the required amino acid, the mutation plates do not. The degree of recovery depends on the type of medium in which the bacteria had been grown before treatment. It might well depend also on the type of treatment.

This finishes what I wanted to say about mutagen specificity. I have shown by examples that observed mutagen specificity need not reflect specificity of reaction between mutagen and gene, but may occur at any one of the many steps that together form the process of mutagenesis. If one is interested in the chemical reaction between mutagen and DNA, all these other sources of specificity are sources of error to be avoided. If one is interested in the process of mutagenesis as a whole they are tools for analyzing its successive steps.

This leaves us, then, with the specificities which really seem to occur at the level of the DNA, and I think there we have the best example in Dr. Benzer's work on phage. He is going to report on that.

Benzer: My attention has been restricted to a very small portion of the genetic structure of T4 phage, the *r II* region. By very simple methods it is possible to examine the location of mutations within this region, with a very high degree of resolving power, so that we can draw a magnified map and tell if a mutation is at this point or at another point only one nucleotide step or more away. To measure mutation rates at various points is simply a matter of isolating a lot of *r II* mutants and seeing where they occur.

The first results I want to show are obtained with "chronic" mutagenesis, that is to say, spontaneous mutations. The result is that mutations are distributed over a large number of different sites and in a nonrandom way. This is evidence for higher mutability at certain points. If you get many mutations at one point, over and above the number expected due to a random distribution, this point is called, for want of a better name, a "hot spot."

Figure 21 shows the spontaneous mutation rate at different points

Figure 21. Topographic map of the *r* II region for spontaneous mutations. Each square represents one occurrence observed at the indicated site.

of the structure. It represents the distribution of approximately 2000 spontaneous mutations.

As far as the sequence of sites is concerned, this is known only segment by segment, that is, the mutations of one segment are ordered with respect to those of another segment, but within a segment the order is not yet established. Some sites which are empty in this figure are known from other mutations, but the occurrences shown are restricted to spontaneous ones.

What is striking is the two very prominent hot spots; at one site, 500 recurrences have been observed. There is only one site in the entire structure that has that degree of mutability. There is another one that has about half the mutation rate, and there are spots of decreasing "temperature," coming down to sites at which only a single mutation has been observed, and then other sites which we know must very well exist but have not yet been observed.

Lederberg: How many sites are there?

Benzer: This map contains 308 known sites in the two cistrons of the *r II* region.

Lederberg: How many nucleotides?

Demerec: Where is the break between A and B?

Benzer: The number of nucleotides is not known, but it can hardly be more than a few thousand by the most conservative estimate. The break between the A and B cistrons is shown in the figure. From the beginning to the broken line is the A cistron, and the rest is the B cistron, which is about half as long. The B cistron also has about half the total mutation rate, but this correlation with size is almost meaningless by virtue of the fact that half the total mutation rate of the whole B cistron resides in one single spot.

From the statistics of this distribution one can estimate how many more sites there must be at which spontaneous mutation has not yet been observed. This can be done from the coolest spots, taking the sites of one occurrence, and the sites of two occurences, on the assumption that these represent the lowest mutable class of sites, and you can calculate the number of zeros there must be. This comes out to be another 120 or so. This is, of course, a minimum estimate because many of the cools may well be slightly warm spots. In any case, the total mutation rate for *r II* mutations must be a composite of at least 400 sites.

The mutabilities of the various sites cover a range (at least) of 500 to 1. So far as the distribution of these sites throughout the structure is concerned, there is no evidence of any large quiet regions where mutations are rare.

Using recombination frequency as a measure of distance, one can try to correlate the distance between two mutations measured in the recombination units with the number of intervening sites. As far as this goes, there is no apparent large chunk of the structure which is quiet.

In the map shown (Fig. 22), segments are drawn proportional to the number of sites known in that particular segment. These segments are arbitrary, being determined by the end points of the various deletions used to map or define the various segments. But they define the sequence of the respective portions of the map.

The figure shows a series of mutants that were mapped by Chase and Doermann (10), without reference to the deletions at all, just by recombination frequency. They are spaced according to the recombination frequency. The first thing to see is that the sequence correlates precisely, without exception, to the sequence determined by segment mapping. Down below is another group mapped by Edgar, Feynman, Klein, Lielausis, and Steinberg, also without reference to deletions. Again, there is a perfect correlation as far as sequence is concerned.

From these data, one can make a judgment as to the existence of quiet regions. If they exist, they would show themselves by discrepancies in spacing of mutations in the two kinds of maps. If there are any they obviously don't involve any enormous part of the structure. Certainly, at the level of fine detail, there is a lot of very close clustering of neighboring sites, but that is something else again.

I mention this because the question of quiet regions came up in Dr. Demerec's talk, and there have been reports of large quiet regions in Neurospora, also. This doesn't seem to be so in the case of *r II* mutants. It is important to point out that in this system the function under study is an expendable one, so that any mutation leading to loss of the function can be detected. In some other systems only a special class can be seen, for instance, those in which one functional protein is altered to a different (but also functional) form. In such a case, one would expect quiet regions.

Getting back to mutation rates, what you measure as the rate of production of *r II* mutations is a composite of at least 400 different individual sites that are contributing to this rate. The way to measure mutation rates in *r II* mutants is simply to put into a tube some bacteria plus a few phage particles of the standard type, so that the chance of introducing a mutant is negligible, let them grow up into a lysate, and then measure the fraction of particles that are mutant.

The proportion of mutant particles actually measured is the mutation rate times the number of generations, which gives a number about

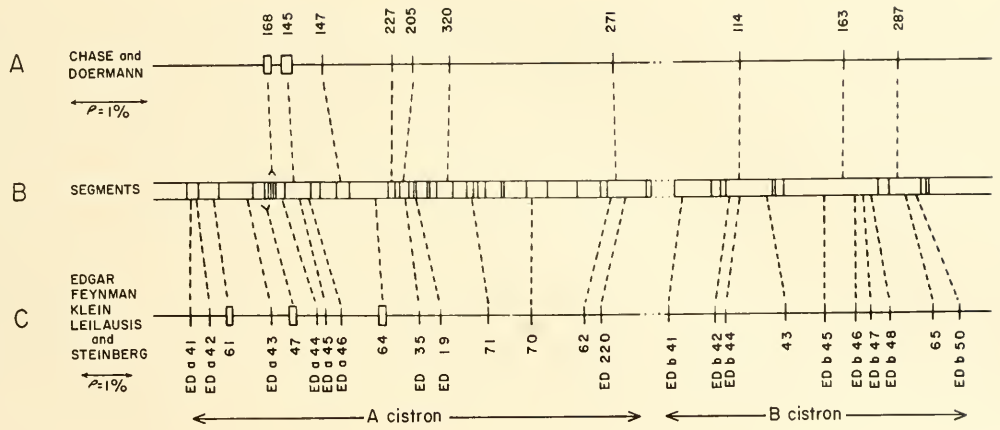

Figure 22. Correlation of the results of deletion mapping with the conventional method and examination of the map for quiet regions. (A) The map constructed by Chase and Doermann (b1) for ten *r* II mutants of phage T4B, using quantitative measurements of recombination frequency. The interval between adjacent mutations is drawn proportional to the frequency of recombination in a cross between the two. (C) The map constructed in similar fashion by Edgar, *et al* (personal communication) with *r* II mutants of the very closely related phage T4D (using a procedure that gives somewhat higher recombination frequencies).

All of these mutations have also been mapped by the deletion method and dotted lines indicate their locations in the various segments (B). The length of each segment is drawn in proportion to the number of distinct sites that have been found within it.

20 times higher than the probability of mutation per replication. This number for *r II* mutants, or for both cistrons together, is approximately 2×10^{-4}. It is the custom to call this the "mutation index." (Divided by 20, that would correspond to a probability of 10^{-5} per replication of getting an *r II* mutation, added up over all these sites.)

The total forward mutation index, which is a composite of many sites, is 2×10^{-4}. This means that the hottest spot in the map, at which 500 recurrences were observed out of about 2000, accounting for one fourth of the total mutation rate, has a mutation index of 0.5×10^{-4}.

Freese: Excuse me, but is that per replication?

Benzer: That is mutation index as defined here. What is actually measured is $\frac{M}{N}$. I am using the same measure throughout, so that the comparison, in talking about forward and reverse mutations, will be valid. The conversion factor to frequency per replication will be constant throughout.

The range of forward rates observable is limited since the coldest spot observed is determined by how many mutants you have mapped. The coldest spots here would have zero occurrences out of 2000, or a mutation index of less than 1×10^{-7}.

If we take each mutant and measure its reversion rate, we can observe a much larger range because we can select for reversions. To measure reversion rate, you do exactly the same experiment, except that you now start with a few mutant particles, so the probability of adding revertants to the tube is negligible. Let them grow up (using a bacterium, where there is no selection between the mutant and the standard type) so they develop into the same size population as before, and you now measure the fraction of revertants. If you do this with various *r II* mutants, you find mutation index values which go from less than 10^{-9} up to as high as 10^{-1}.

So far as I can see, there are about as many grades in between as one can determine experimentally. It is difficult to distinguish a factor smaller than 2 because of statistical fluctuations.

Lederberg: What is the smallest possible number?

Benzer: The smallest nonzero number is 10^{-9}. This is limited by the sensitivity of the test.

Lederberg: There is no obvious gap?

Benzer: That's what I mean to say. I haven't seen any obvious gap. There are many different rates. The other point is that there exist reverse mutation rates which are higher than for the hottest forward

spot; in fact, there exist here mutants which have a reversion rate of orders of magnitude higher than the total forward mutation rate, which is a composite of at least 400 sites.

The fact we are faced with here, then, is that there exist, among reverse mutations, types of events which are excluded among the spectrum of forward mutations.

Lederberg: Are you making a point of that?

Benzer: Yes, but give me more time. This immediately raises the question of whether reverse mutants cannot themselves represent a composite of several different possibilities.

There are many precedents for this in many genetic systems, where suppressor mutations, at a different site, produce a false revertant. In the case of *r II,* Feynman has actually demonstrated suppressor mutations within the *r II* region. If you take a mutation at a particular point, you can have other mutations at any of several points which will suppress the effect of the first one so that the first mutation rate you measure in such a case will be a composite of several.

Zamenhof: Did you ever observe mutation from nonhot to hot spot?

Benzer: Nonhot to hot? You mean a mutation, a change in a cold spot so it is now hot?

Zamenhof: Yes, and also vice versa.

Benzer: I can give you the opposite. I can modify the structure to get rid of hot spots.

Lederberg: Another more obvious point I was going to ask is, if you take the temperature at the spots you actually find, are they unlikely to be found in an extremely unstable state?

Benzer: The point I'm trying to make is that when we're mapping mutation frequencies in a forward direction, we're not looking at a random selection of the types of molecular events which can happen in a piece of nonsense genetic material, but we're looking at a structure which has evolved in such a way as to eliminate the hot things.

What this suggests is that those hot spots which still remain are simply a matter of evolution not having reached its culmination. This might suggest that in Dr. Demerec's system, where he doesn't find hot spots, the bacteria have evolved a little further than phage has, and have gotten rid of them.

Freese: I would like to mention in this connection that phage contains, first of all, hydroxylmethyl cytosine instead of cytosine, and second, the hydroxylmethyl cytosine is glucosylated, and not all of the HMC bases are equally glucosylated. I have reason to suspect

that some of the hot-spot effects in these phages are due to differences in isolation.

Benzer: What is your reason for this?

Freese: Hydroxylamine reacts with hydroxylmethyl cytosine more easily than with cytosine. It does not react with methyl cytosine. It does not react with pseudo-uridine but it does react with uridine. In other words, whenever one puts on the 5-position of cytosine a methyl group or a sugar, it reacts less; the reaction goes down. I have not yet measured—because I have not had the compound—the reaction with the glucosylated HMC and the nonglucosylated HMC, but because we have certain mutagenic results which would indicate that there is a difference, I suspect that this possibility should be looked into.

Lederberg: There is a way to test this.

Benzer: This specific point?

Lederberg: Yes, I think so, that is, to compare the mutation spectrum of T2 with T2 that has once been crossed with T6. Has that ever been done?

Benzer: No. I am in the process of comparing the spectrum of T4 with the spectrum of T6, to see whether they differ. As far as it has gone, I can say that the two major hot spots that T4 has are still present in T6, but appear to have different relative mutation rates.

Lederberg: It might be more appropriate to use an F_1 hybrid or back-cross stock which has the same DNA as T-4.

Benzer: Yes, I know. The difficulty with that is that you have no guaranty that the glucosylation really applies in detail within the *r II* region.

Auerbach: Is it possible to test the influence of the genotype of the bacterium on this spectrum?

Benzer: My only answer to that is that I have enough troubles.

Auerbach: But on this depends the interpretation of the hot spots, doesn't it?

Benzer: I don't see why, because the whole beauty of the system, if it has any beauty, is that it is restricted to one type of mutant, one particular region of the phage, and everything else is kept constant except the thing that you're looking at. What I'm interested in is the internal specificity of the structure.

Auerbach: Yes, that may be too troublesome, but in bacteria, mutagen specificity is under the influence of the residual genotype. In your case, it might be under the influence of the bacterial host strain.

Benzer: In which way could you expect that to modify the result?

Auerbach: I don't know which way, only I should like to know whether it does modify it. It should be excluded, I think.

Neel: Yes, I agree with Dr. Auerbach. If, in another strain, you found that hot spots were differently situated, this would certainly influence your interpretation that the hot spots were a way station.

Benzer: All the experiments I did were in one bacterium. If I do one experiment in each of the different bacteria, I am distributing all my effort in the wrong direction.

Freese: It could be that the DNA polymerase has a specificity to certain base combinations.

Zamenhof: Perhaps I could mention a few words about the hot spot in bacteria, where such experiments can be performed with transforming principle; in fact, we are doing it now, but no results as yet. We have an unstable gene in which we have produced a hot spot or instability by a mutagenic agent. We are extracting transforming principle from it, and we are now trying to transform a stable strain, which is a different strain, by means of this DNA. If we can, that means that the instability was due to hot spots in the DNA itself. If we do not succeed, then, of course, negative results do not mean much; however, if they are taken at face value, it would mean that it was the environment of the spot or DNA which produced hot-spottedness and not this particular spot of the DNA itself.

Benzer: Of course, everything that happens is the product of the gene and its environment. The bacterium is the environment of the phage. The point is, in a *given* environment, the different sites in a cistron have different mutational responses. But I would rather change specific parameters, like adding base analogues, than change from one bacterium to another.

Zamenhof: In bacteria, there could be two cases. One could produce hot-spottedness, which would disappear if one removed DNA and by transformation put it in a different environment. But it could be the other case, that the hot-spottedness was not due to a hypothetical intracellular mutagen; it simply was a hot spot in the DNA itself. It is important to differentiate between these two cases.

Benzer: I would like to come back to the question of whether these very high reverse mutation rates are merely a composite of things that may happen at many sites. We test this by the following experiment, which is a good deal of work, but it may be worth it:

We measure the forward mutation rate at a particular site, from the number of recurrences. Now take one of these mutants and from it obtain a revertant. The question is whether this revertant is genuine.

Does it really represent a return to the original configuration at the same site? To test for suppressor mutation, you back-cross to the wild type and see if you can get segregation of the individual mutants. Feynman found this for some apparent revertants, but his revertants were deliberately chosen because they looked false (by phenotype) to begin with. When I did this experiment using only revertants which looked genuine, it failed.

Another is the following one. If it has really returned to the original wild type, it should have exactly the same mutation frequency at the same site as before. This is a very stringent test of the idea that this is a true reverse mutation. This experiment has been done for nine different revertants, and all but one gave exactly the same mutation rate, at the same point as before. Without this test, I would consider the measurement of an apparent reverse rate as unreliable. This is particularly pertinent to experiments on induction of reversion by mutagens. Short of a proper demonstration that you return back to the original type, you do not know whether you are working on a reversible transition or inducing mutations at some suppressor site.

This experiment, when applied to a mutant with a very high reversion rate—one of these rates excluded from the forward class—still gave the same result, so that genetic transitions at a very high rate, which are truly reversible, are possible.

It is quite frequent, and this is especially characteristic of the spontaneous hot spots, that the mutations arising at the same point have different reversion rates. One case has been studied in some detail, at which three different reversion rates were found.

This experiment of forward, reverse, and forward again, has been done with all three types, and in every case we got back the same forward mutation frequency at the site.

As I pointed out, to do this experiment, the revertants which I picked were chosen because they really looked like standard type. To do his experiment, Feynman deliberately picked ones which looked as though they were not a complete return to the standard type, and in his cases he found that the suppressor mutations were at other points.

Auerbach: In Neurospora, Giles (29) got partial reversions which seemed to have occurred at the same site, and yet were not full reversions. Of course, the structural analysis can't be so fine with Neurospora.

Benzer: One of the revertants we studied appeared to be not quite identical to the original standard type but had a very slightly mutant

character (judged by plaque morphology). In this case the experiment gave the opposite result, that the forward mutation frequency at this site was much less in the revertant than in the original standard type. In other words, by this trick of going forward and back, it is possible to eradicate partly at least this hot spot.

Lederberg: Assuming you had a specific suppressor of the very frequent reversion at that site, right?

Atwood: By two steps you arrived at this figure?

Lederberg: You had a third state at that site?

Benzer: This experiment does not prove that. It could be that the false revertant is due to a specific suppressor at another site. In that case, mutations at the original site could appear to be observed as a result of reversion of the suppressor.

Lederberg: No, that's the mutation you're measuring there.

Benzer: It might be interpreted in more than one way. My point is that the experiment doesn't always come out to give a demonstration of true reversion.

Deletions, as in the case of point mutations, do not occur in a random way throughout the structure.

Figure 23 shows some of the deletions; it does not show their distribution, however. The deletions here have been chosen to be all different and are the ones used to define segments of the map. It shows at least the number of different end points which deletions can have. There are many other deletions, several hundred more, that are available, but they are not shown here because they do not overlap in just the right way to be used to define the sequence of the segments. These are almost all from spontaneous mutations. A few have been obtained by Freese (23), by the use of low pH and high temperature treatment.

This figure does not illustrate the frequencies of various end points, which are in fact nonrandom. One of the striking things is that there is a kind of polarity. Many cases are observed where "deletions"—I use the word in quotation marks although we have evidence that they are deletions—begin at one point, and proceed all the way to the end of the B cistron. But there is no case observed, so far, which starts at a point and proceeds all the way to the left. This could, of course, be an artefact due to what there is outside the *r II* region, if there were an expendable structure on one side and, on the other side, one which were not expendable, so that one kind of deletion would be lethal. Some end points recur with high enough frequency so that they clearly represent hot spots for deletion. For instance, the joint be-

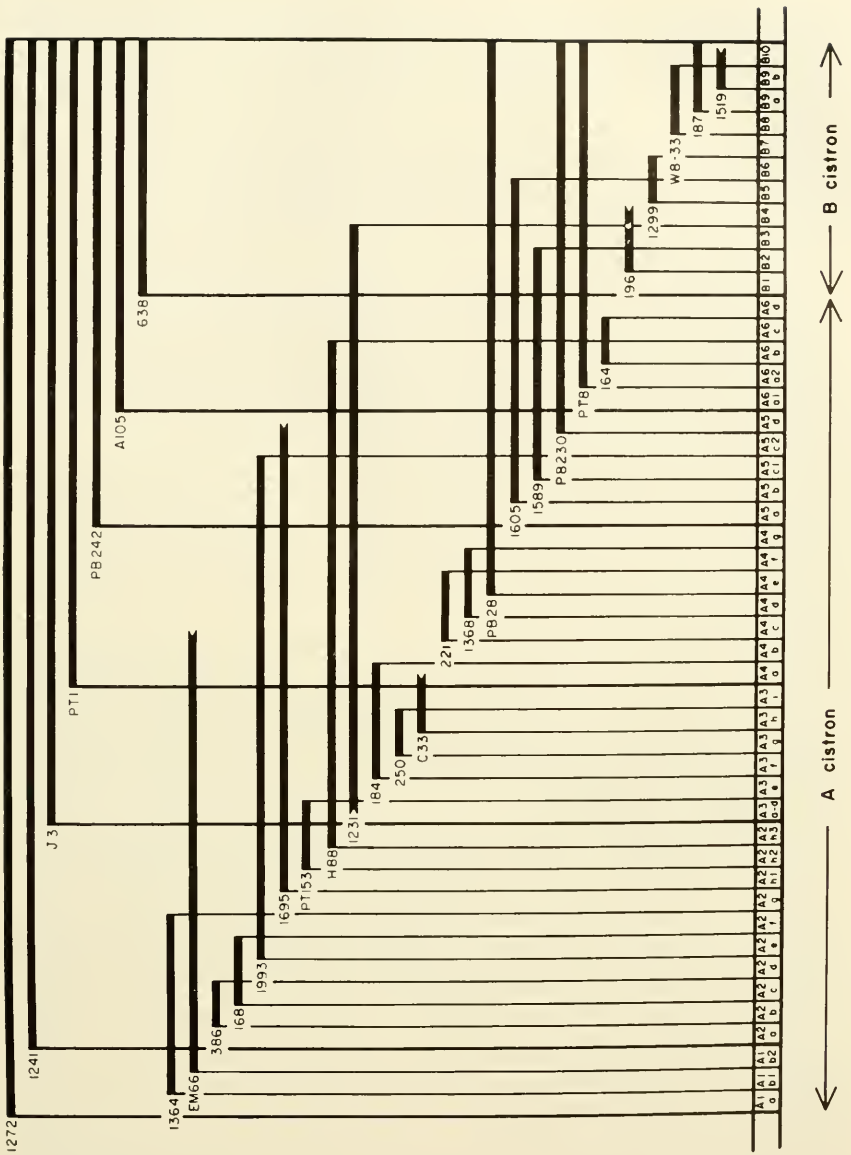

Figure 23. Deletions used to divide the *r* II region into 47 segments. (Some ends have not been used to define a segment, and are drawn fluted.) The A and B cistrons, which are defined by an independent functional test, coincide with the indicated portions of the recombination map.

tween the A and B cistrons must be such a hot spot, because this type of mutation which cuts out the whole B cistron has been observed no fewer than ten times. This is also true of many others, including small deletions.

Therefore, there are hot spots for the beginning and end of a deletion. You can find nests of deletions within deletions.

Novick: Do the spontaneous hot spots tend to be in the center of the deletion hot spot?

Benzer: No. There is no apparent correlation between hot spots for point mutations and hot spots for deletion end points.

Lederberg: Do you want to say just that—or that there is a tendency for the breaks to occur, and then you get all combinations of segments between those points of breakage?

Benzer: Yes, many combinations.

Lederberg: To generate the type of segment you have, would it be sufficient to give the frequencies at which you find the beginning and end and put them together in an appropriate combination, or, if it starts here, does it have a particular reference ending there rather than here?

Benzer: I think I would want to say both. There are cases of the beginning correlating with the end, and those are the ones I'm talking about here—recurrent, identical deletions. But there are also many cases where the beginning points look the same but the end points are different. You see I can give you almost anything you want. I've got a trunkful of mutants.

Freese: How hot is such a beginning point or an end point when compared to the hotness of point mutations?

Benzer: We can estimate among the same set of mutants studied for point mutations, where the hottest spontaneous spot has 500 recurrences, the hottest deletion has of the order of 10. Approximately 1 in 20 spontaneous mutants is a deletion.

Auerbach: But what if you take an end point only, not the whole thing? It must be hotter.

Benzer: It's not much different, since in the hottest cases the end points are mostly the same.

Lederberg: You define the terminus of this particular class of deletions at this point. What is the temperature for point mutation at this particular point?

Benzer: There is no obvious correlation. Of course, it must be understood that the terminus of a deletion is only defined in terms of two sites. All you can say is that a deletion ends between one and the

other. But the ends do not seem to correlate with hot spots for point mutation.

Auerbach: Are there preferred size classes of deletions, or do you get all classes, more or less?

Benzer: It is difficult to answer the question in those terms. There are certain deletions which tend to recur. The small ones tend to recur. Then there are big ones which tend to recur, and also all kinds of sizes in between.

Neel: If I understand Dr. Auerbach's question, it implies that if you plot the lengths of the deletions, you observe a normal curve.

Benzer: I'm sorry, but that has not yet been done. I hesitate to predict just what it would look like. The first step has been to carve up the map with big deletions in order to obtain all the sites. Now that we have identified many sites, we can use them to determine the termini of all the deletions much more accurately than could be done before.

Auerbach: I wonder if it shows anything about the way the phage chromosome is coiled? You may have loops.

Benzer: Perhaps so. Let us turn to the results on induced mutations. Here we have hot spots too. A mutagen may increase the frequency at particular sites by a factor of 10,000 or more, while leaving unaffected some other sites.

Some mutagens that have been examined in this way so far by various people are: nitrous acid, ethylmethane sulfonate, 2-aminopurine, 2,6-diaminopurine, 5-bromouracil, 5-bromodeoxycytidine, proflavine, ultraviolet light, heat at low pH, and hydroxylamine (7,8,9,24).

Auerbach: The ethylmethane sulfonate, from what Dr. Freese said this morning, was applied *in vitro*.

Benzer: Yes, some of the mutagens such as nitrous acid were applied *in vitro* and others *in vivo*.

Lederberg: For the record, you might say what you mean by *in vitro* and *in vivo*.

Benzer: In vitro means that you take phage in a test tube without the bacteria, treat it with the agent, and then plate it on bacteria to see if you have any mutants. *In vivo* means that you infect the bacteria with phage and then add the mutagen while the phage is multiplying.

The figure shows the distribution in the *r* II region of mutations induced by some of these mutagens. The top line is for spontaneous; on successive lines are nitrous acid, ethylmethane sulfonate, 2-aminopurine, 2,6-diaminopurine, 5-bromouracil, 5-bromodeoxycytidine, and proflavine.

In each of the induced spectra there is, of course, some spontaneous background which has to be subtracted out.

Magni: In this map have you not yet subtracted the spontaneous mutants?

Benzer: I can't, because I don't know which ones they are, but in analyzing it statistically, you have to not take seriously recurrences at a site where they can be largely due to spontaneous mutation. On the other hand, if you have a site where there are 8 or 10 recurrences with nitrous acid and only one spontaneous out of 20 times as many mutants, the probability is extremely high that that is really a nitrous acid hot spot.

Lederberg: I think it would help if you indicated what weight to give the different lines, because they do represent different mutants.

Benzer: Yes, the number of mutants on each line is different. It is very difficult for us here to assess the significance at the various points. It would take too long. If you look at the coincidence of hot spots, i.e., look at a point which is a hot spot for nitrous acid, and ask whether or not it is a hot spot for other mutagens, you can give each such spot a set of quantum numbers, if you will. Unfortunately, a clear decision may be limited by the statistics of how many mutants you have actually studied.

You find, for instance, with the ethylmethane sulfonate spectrum, there are 26 of what statistically ought to be significant hot spots, and, out of these, 19 are also present in the nitrous acid spectrum, 6 are also present in the aminopurine spectrum, 5 are present in the diaminopurine spectrum, 7 are present in the bromouracil spectrum, 8 are present in the deoxybromocytidine spectrum, none in proflavine.

One can pick out many, perhaps 25, different kinds of spots having different responses, different sets of decisions as to whether or not they are hot for such or such a mutagen.

Goodgal: Do you have a sufficient number of hot spots with each of the mutagens so you can make—

Benzer: These are the ones I could say something about. The number might be much larger if one studied more mutants.

Atwood: Your matrix may become simpler if you have more.

Benzer: A spot being not hot is also statistically significant.

Atwood: I see. You have omitted those.

Benzer: I call a spot cold if you expect three or five mutations there and find zero. Of course this can be due to chance. Coldness is also subject to statistical test.

Lederberg: Twenty-five? Does that mean there were eight different

eigenstates for the spontaneous mutations? This is about the number of levels that you usually pick up? Or is it just either hot or cold?

Benzer: There are eight mutagens (including spontaneous) that have been studied here. The number of possible combinations is 2 to the 8th power, or 256.

Lederberg: It is either "yes" or "no" for a given agent? You don't quantify the different levels?

Benzer: That is what I would like to do, but the data are not always sufficient. The point I'm trying to make is that there are many kinds of different spots. This is seen in the forward mutation rates of the various spots, in the reversion rates for the various mutants, and in the different patterns of response of various sites to different mutagenic agents. One is forced to think in terms of neighbor interaction between groups of bases, and not simply what happens to a single base pair independently of its neighbors.

Auerbach: Are there any coincidences between patterns of mutagens which are more frequent than expected from random coincidence? Do some of these mutagens have more overlap than the others in their patterns?

Benzer: Yes, of course. Those are the numbers which I was just giving you.

Auerbach: It was so quick that I couldn't follow it. For instance, the diaminopurine and the bromouracil are very similar.

Benzer: Hardly. Diaminopurine has 15 significantly hot spots, of which only 2 have also been found hot with bromouracil.

Neel: How many other bromouracil hot spots were there? What is the rest of it?

Benzer: Two out of 18 bromouracil hot spots.

Auerbach: If you look at the mutagen rather than the spots, are there any mutagens whose similarity of action on these spots is noticeably greater?

Benzer: Yes, there are certain similarities. A striking one is the similarity between ethylmethane sulfonate and nitrous acid which, according to the chemical indications, should act by utterly different mechanisms.

Freese: But they both attack preferentially the CG pair.

Benzer: Yes, that's right.

Auerbach: According to Vielmetter, the action on the guanine is just the one which does not produce mutations in TMV.

Freese: The action on the C is five times higher than that on the A.

Lederberg: Given this much wider range of discrimination, could you now say there are not more than four states at any one site?

Benzer: Yes. Ernst tested this one which I mentioned before. It is a hot spot, but has three different kinds of mutation distinguished by their reversion rates. He tested these for induction of reversions by bromouracil and 2-aminopurine.

Lederberg: Out of how many incidents?

Benzer: Out of how many occurrences at that site? Several hundred.

Lederberg: But was it tested with respect to number of different agents? You see, it is much more versatile.

Freese: I found that one of them can be induced to revert by bromouracil and aminopurine, while the other two cannot.

Lederberg: But there are several recurrences within each of these states.

Novick: His question was, can these states be split? I think that is his question.

Lederberg: I am looking for splitting of the state by looking at several recurrences within them. If that has been done—

Benzer: That is what we are talking about. Reversions can also be split. There are many cases where a mutant reverts to two different classes, one of which looks like the real standard type and the other looks like a partial revertant.

Lederberg: No, that is certainly applicable, but I think that the data could be put to a very sensitive test with the idea that there are really only four states. To take one of your hot spots in forward mutation, this gives you four classes with respect to spontaneous reversions—or, excuse me, three classes—which are presumably the three alternative bases having been substituted at that point. If that is true, these individual states which include several representatives each, several incidents of mutation each, should not be further split by using this large a battery of tests, even though a wider variety of mutants would be split.

Benzer: You mean there shouldn't be any new classes coming up, by using chemical mutagens?

Lederberg: Yes.

Benzer: Actually, the mapping has only now reached the point of getting everything in its proper site, so the examination of all the classes has not yet been done. It should prove very interesting.

Lederberg: Of the four classes of reversions, are there four classes of

mutants, in the first place? You don't have to go this far with reversions. Just measure your reversion rate at the different environments.

Benzer: Of the same mutagen?

Lederberg: I'm going to have to clarify this. This was one of the hot spots you talked about before. Now, when you look at these from the point of view of their reversion rate, you find there are three classes of mutants. There is the class that had a rate of 1000, there is the class that had a rate of 1, and there is the class that had the rate of .03. I would say that we actually split this up into three categories. But it is not a very sensitive test. The test has to do with the level of the reversion rate, but could we further split these up by now applying chemicals?

Benzer: I see what you mean. What appears to be one class could be split into different groups.

Lederberg: Right, by the use of a battery of mutagenic agents. If, in fact, they cannot, I think that it would be very strong evidence that they are really uniquely just three states, and it would be the strongest support I could think of from this kind of datum that we are really talking about a—

Neel: How would partial reversions within this cistron fit into your argument, Josh?

Lederberg: I would say a partial reversion, if it is a reversion, could not take place when you already know the rates; that is, you should be able to list four alternative bases at a given site. In some cases, three of the substitutions will have the mutant phenotype and the original will be the wild type. Then you will have three reversion rates and the wild type. In the cases that you referred to, where you have a partial return to the wild phenotype, it may be that the substitution of one base does not give you the mutant phenotype but some intermediate phenotype. But adding it all together, you should not find more than four states at any one site.

Auerbach: But, if I understood him rightly, Seymour thinks that the hot spots of his mutagen are influenced by a position effect of the neighboring nucleotides. Would that not be influenced by an exchange in the relevant base pairs so you might expect a splitting, simply because the AT interacts differently with a neighboring GC?

Lederberg: That is why we can't predict what these numbers will be in advance, because we don't know what the neighbors are and what effect they have on the spontaneous reversion.

Auerbach: No, I'm sorry. May I show you what I mean? If you have a neighbor here and a neighbor here, if your original neighbors

are 1 and 2, and the original was AT and responded in a certain way, giving a certain mutation pattern with your chemicals, when you change the neighbors this may alter the pattern.

Lederberg: Indeed it should, and there should be two alternatives, you see. There should be no more than four patterns all together. Using the very subtle criterion—and you have indicated how subtle it is, because there are no fewer than 25 conceivable possibilities of the pattern of response to the whole battery of tests—

Goodgal: But his prediction is that there will be more than four.

Atwood: Oh, no!

Lederberg: If there are more than four, they are not nucleotide substitutions.

Zamenhof: The effect of neighbors, Seymour, could be the presence of clusters of purines or pyrimidines which may exist in nucleic acid. That is why I asked you the question before as to whether you observed any mutation from coldness to hotness. Is it as easy to produce it as it is an ordinary mutation, or do you need several mutational steps to change this pattern from coldness to hotness? If you find that you never observe such mutations, that may mean that the change necessary to produce this effect may really be a very complex one.

Benzer: I think what you are proposing is that we start with the standard type, and you want to pick up a mutant which is going to affect the mutation rate at a neighboring point—is that right?

Zamenhof: No, I am asking how easy is it to change the chronic cold into chronic hot. Is the change as frequent as ordinary mutation?

Benzer: How do you propose to change it if not by mutation at other points?

Zamenhof: Yes, but perhaps you need mutation at many points, and therefore such a change from cold to hot would be rare.

Benzer: If these changes themselves give you a mutant, you cannot detect the occurrence of an additional adjacent mutation, except by testing every single particle genetically against every other one. If they themselves do not register as mutants, they are invisible.

Zamenhof: That's what I mean, invisible mutation.

Atwood: You could guess that a point might be subject to invisible mutation by seeing that its mutants form only two reversion classes instead of three. Then, you could infer a fourth state that is wild type, like the first one. Both wild-type states should occur among the revertants, so if a case of this sort were found it would be worthwhile screening the revertants for hotness in nearby sites.

Benzer: If I understand you, Dr. Zamenhof, the reason you propose this is as a direct demonstration of neighbor interaction.

Zamenhof: Yes, that is the reason. Just how many things do you have to change at once to change mutability of any particular spot? Is it directed by one neighbor, two neighbors, or the whole cluster?

Benzer: It might be feasible to do something like this in dealing with reversions, using linked mutants or something you can turn on or off, something with an intermediate phenotype. It might be possible.

Lederberg: You do have these incomplete reversions, where you know you have more or less wild type, and presumably at least one base substitution, and one could inquire whether this in itself has had any influence on the spectrum of the nearby loci. I'm not proposing that you do it. I really don't see how it can tell you that it is a string of adenines or what.

Benzer: Since the bonds between adenine and thymine are weaker than the bonds between guanine and cytosine, if you have a stretch of adenine-thymine pairs, it will be much easier to make a purine-purine pair, and thereby a mutation, than in a part of the chain which is held together much more tightly. That would illustrate one type of neighbor interaction.

Atwood: To return to your conjecture that the hot spots are left over from a trend toward elimination of hot spots in evolution, I think the idea that you wouldn't expect to go to completion in this trend is more likely since the hot spot depends on an array more or less around the region—I mean, we don't know how far from it, but it depends on the entire sequence, and so does the rest of the phenotype that is subject to selection. Then you always have to have a compromise. If it is bad for the thing to have a hot spot, it is also bad for it to change the sequences so as to avoid the hot spot, and you're going to end up with a compromise between the number of hot spots and the phenotype.

Benzer: Yes.

Lederberg: Or, alternatively, if you had the wild type so hot that you had ten thousand out of a million mutants at one culture, it would take a selective advantage on the part of the wild phenotype to maintain itself against that mutation.

Demerec: I'm wondering if you are familiar with Streisinger's work or his claim that he found five different states in a single site.

Benzer: Yes.

Goodgal: How many mutants did he test?

Benzer: He seems to have four distinguishable mutants at the same site.

Goodgal: Out of how many?

Benzer: I'm not sure. It is a different kind of mutant, the host range mutation in phage.

Goodgal: From five different levels out of 50, or four different levels?

Benzer: I seem to recall that some of them were obtained by a kind of reversion at the site, so that they are not all obtained as forwards. I think he had forward mutations, and then several revertants with different degrees of return to the original type. How many of these could possibly be suppressor mutations, it is a little difficult to conclude definitively.

Atwood: Just one more question. Is the small number of reversion classes the principal reason you have for thinking that the hot spot is not a composite of more than one position?

Benzer: Not at all. Position is based entirely on the absence of recombination.

Atwood: Do you have any other reason besides the small number of reversion classes for thinking that there is not a composite of several base pairs unable to recombine within a hot spot?

Benzer: The reasons for believing two mutations to be at the same site is the failure to detect recombination between them, even in a frequency that would be expected if they were only one nucleotide apart.

Atwood: One can just as well say that there are variations along the chain, in frequency of recombination, that account for this, then.

Benzer: Yes. One could imagine regions where recombination is suppressed. Anything I show as a hot spot is always subject to the possibility of being split up by a more sensitive test, and if I indicate two mutants at the same point, it only means that I have not succeeded so far in demonstrating, by recombination, that they are different. The only thing you can demonstrate in recombination experiments is that two mutations are at different points. You cannot prove they are the same.

Lederberg: Would you dispute the former hypothesis that the individual units of mutation are nucleotides, and that there are definite spacing factors, so recombination takes place between groups of three nucleotides? That would give you the number of varieties of configurations you want for the different states that you described. It might make some physiological sense, too.

Benzer: I don't think I can really prove that that is not the case, although I don't believe it for a moment.

Lederberg: I don't either, but I would just like to know how we dispose of it.

Benzer: If one had a good estimate of the correlation between re-

combination frequencies and actual molecular distances, one could say something from the number of sites. You would have to have saturated the map at least within a factor of 3 to make this decision.

Lederberg: Exactly. My impression was that you had not run the map down to that level as yet. You could think that the map was devised in triplets of nucleotides, and if recombination took place only within the triplets—

Neel: Seymour, if there were, in fact, three different reversion rates at a well-studied locus which could be related to purine-pyrimidine substitutions, doesn't it follow that for each reverse mutation there should be two mutations, assuming a randomness, to an alternate purine or pyrimidine which would not result in a change of phenotype but would alter the rate of reverse mutation. Is it feasible to attack it from that angle?

Benzer: You want me to look for a change from one mutant to another one that looks just like it, at the same site?

Neel: That's right. But there is a change in the mutability state.

Benzer: That is not an easy experiment.

Summary of Discussion

Demerec: The fact that Dr. Auerbach led the discussion in accordance with a well-organized outline makes my assignment a great deal easier. I do not intend to repeat in abbreviated form all the topics covered by Dr. Auerbach but shall stress a few of the special problems that were taken up.

Before going into a discussion of these problems, however, I wish to say a few words about Dr. Auerbach's introductory statement with which I am in full agreement. She expressed concern lest the beautiful findings about the nature of the genetic material obtained in chemical studies of the transforming principle, of phages, and of viruses should make us forget that this material resides in the cell where a great many things happen that would not happen to anything *in vitro*. We should try to avoid overemphasis on the results of work with simple organisms to the neglect of prior information accumulated during more than half a century of genetics research which, after all, forms a solid foundation for the structure of knowledge we are trying to build. We should be mindful that in many of the problems now being considered a backlog of reserve information already available might be utilized to great advantage.

In the brief review of the study of chemical mutagenesis it was pointed out that attempts to induce mutations were first made with

pharmacological chemicals: mustard gas, because it resembles X-rays in the kind of burns it produces; alkylating agents, because of their carcinostatic properties; various alkaloids, because of their medicinal uses. Other substances were then tried, and are still being tried, because of their ability to fit into theories of how a mutagen may act. Formaldehyde was used because of the belief that a protein is the carrier of genetic information. Since the discovery that a nucleic acid plays this role, tests have been made with various substances that react with amino acids, particularly certain analogues of purine and pyrimidine. In the history of chemical mutagenesis, the wrong theories have often led to the selection of good mutagens, and this warns us to be cautious. It means that if we have a theory about mutagenesis, choose a chemical on the basis of this theory, and find it to be mutagenic, we do not necessarily prove the theory.

The discussion of the action of mutagens brought out the fact that, in two stranded phages as well as in bacteria, treatment with a mutagen produces mosaic plaques or colonies, indicating that both strands of DNA give rise to progeny. Zamenhof found no mosaics among auxotrophs of *Escherichia coli* induced at frequencies up to 10 per cent by heat treatment and interpreted their absence to mean that under the conditions of the experiment the heat treatment eliminated one of the two DNA strands. Lederberg expressed the opinion that the bacterium is far too complex a genetic entity to be suitable for experiments on mutagenesis and pointed out that the available information about the structure of DNA cannot eliminate the possibility that at some stage of the bacterial life cycle DNA may be single-stranded.

During the discussion of the action of the mutagens Freese presented his ingenious models interpreting the types of change induced in phage T4 DNA by treatment with purine and pyrimidine analogues and certain other potent chemical mutagens. Mutations induced by a direct effect on DNA may be due to mistakes in incorporation or mistakes in replication. Two types of changes may occur: transitions, that is, replacements of a purine by a purine, or a pyrimidine by a pyrimidine; and transversions, that is, replacements of a purine by a pyrimidine or vice versa. The discussion clearly indicated that Freese's models constitute a good start in the right direction, although most of the processes involved in mutagenesis are too complex and still not sufficiently well known to be explained in terms of simple models. In organisms higher than phages, where the genetic material seems to be better protected against changes in its immediate environment, the complexity of mutagenesis is likely to be greater. Freese, in the course

of the discussion, supported the fairly strong possibility that, at least in higher organisms, many mutations are due not to a direct effect on DNA but to an effect on either nucleotides or proteins.

I was greatly interested in Benzer's presentation of the latest data relating to the "topography" of the *r II* region of phage T4 and should like to comment on it in more detail, because it deals with problems I am much concerned with at present. There are more than 2000 mutants of separate origin representing that region and about 300 independent sites have been identified. Since calculations indicate the existence of about 400 sites, we can expect that at least 100 remain to be discovered. Mutants of the *r II* region belong to two complementation groups, A and B, and thus the region includes either one or two gene loci, depending on the definition of a locus. It seems to me that the experimental evidence, rapidly accumulating in work with phages and bacteria gives strong support that a genetically identifiable site corresponds to a nucleotide pair of the DNA molecule of a gene. If we accept this interpretation, it can be estimated that the *r II* region contains about 400 nucleotide pairs—provided, that is, that every nucleotide pair is a part of the genetic code. At last year's session of this group Levinthal estimated that the gene locus controlling the phosphatase system in *Escherichia coli* comprises approximately 2000 nucleotide pairs. If both these estimates are reasonably accurate, they indicate either that the gene loci of *E. coli* are longer than those of T4 phage or that different genes in different microorganisms vary considerably in length, or both.

The most striking feature of the topographical map of the *r II* region is the identification of two "hot spots," one in section A and one in B. The hot spot in region B accounted for almost 500, or about 25 per cent of all the mutants found, and the other hot spot for almost 300, or 15 per cent. Thus about 40 per cent of all mutations occurred at these two locations. How can we explain this observation? Obviously, the instability can't be due to the constitution of the nucleotide pair present at a hot spot site in the wild-type gene because, since there are only four kinds of nucleotide pairs, hot spots would then be frequent. So it must be the environment of the site that affects its mutation frequency. The position of a nucleotide pair, therefore, or even more probably its relationship with surrounding nucleotide pairs determines its pattern of reduplication, that is, whether it will be reproduced or replaced by a certain other nucleotide pair.

There is evidence—and Benzer discussed such data in connection with his material—that reverse mutants may be due to reverse changes

at the original mutational sites. But there is also evidence, from the work of Feynman, that reversion may be produced by a change at some other site of the same gene locus (a suppressor). Technically, it is very difficult to determine whether a reversion is a true back mutation or a suppressor mutation, and therefore the relative proportions of the two types of change cannot be estimated. The combined evidence seems to indicate, however, that a gene locus is a highly integrated system and that damage to one part can be repaired by modification of some other part.

Both these phenomena, hot spots and intragenic suppressors, remind me of two problems that were extremely absorbing about twenty years ago, namely, the question of position effect and the concept of the gene balance. They dealt with a higher level of genetic organization than those we are considering here, but the similarity suggests the possible existence of a basic and general principle. If that should be so, let us hope that work with microorganisms, which can be carried out closer to the molecular level, will be more successful than Drosophila research was in discovering the mechanisms responsible for these processes.

I wish only to add that this session's discussion clearly pointed up the fact that we are now in a very exciting stage of the work with microorganisms, fully as exciting as the work of almost three decades ago with Drosophila. I am certain that it will uncover at least as important results for our understanding of the basic problems of genetics.

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MUTAGENS CURRENTLY OF POTENTIAL SIGNIFICANCE TO MAN AND OTHER SPECIES

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The title of this presentation refers to "man and other species." However, I shall confine myself to man, and to the question whether substances which have been shown to be mutagenic in other organisms and about which we have heard a great deal here yesterday may have any significance at all as mutagens in man. As you will see, there is very little concrete information, so that what I might be able to accomplish today, with your help, is to indicate some areas where it would be useful to have more data and to suggest what kinds of investigation in man might be initiated to clarify the situation further.

First, I shall make some general remarks related to potential mutagens in man, that is, to drugs in man and to the exposure of man to a chemical environment. Second, I shall present a brief survey of drugs and other substances to which man is exposed. Third, I shall indulge in a rather detailed discussion of caffeine, which, as I shall try to show, is a contender of first importance among possibly significant chemical mutagens in man. Finally, I would like to initiate a discussion of what specific steps should be taken to gain more information about this problem and to minimize any genetic hazards that may be shown to exist or to be likely in consequence of man's exposure to chemical agents.

In recent years there has been, I believe, some overemphasis on the genetic hazards of radiation, accompanied by an underemphasis on the possible genetic hazards of chemical mutagens. I can illustrate this by two quotations indicative of a belief that man is in some way protected against chemical insults.

Haldane (30) wrote in 1954, for example, "radiation and high-speed particles are efficient because they can generate such mutagenic substances inside the nucleus, whereas, if they (mutagenic substances)

are injected, or given in the food, they have to pass through a number of membranes and through living substance, which destroys them to a large extent."

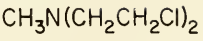
Neel and Schull (42), at about the same time, wrote: "Moreover, because of the elaborate mechanisms which maintain the chemical constancies of the human body during life, it seems unlikely that exposure to various chemical agents exerts a very large influence on human mutation rates, but the situation with respect to certain types of radiation which can penetrate the body with ease is potentially quite different."

At the other extreme, I may point to Barthelmess (5), at the Botanical Institute in Munich, who three years ago wrote a review on the genetic hazards of drugs in medicinal use. After presenting a very thorough literature survey, he concluded that all drugs may be potentially hazardous to the germ line of man. Therefore, he argued, the routine toxicity testing of all drugs should include assays of mutagenic activity before approval is given for use in the human population.

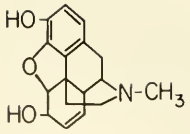
Just as it may be dangerous to underestimate the possibility that chemical agents can cause genetic damage in man, so is it probably wrong to cry wolf about every drug to which we are exposed. I shall raise for discussion later the practicability of developing meaningful tests for mutagenic activity. The real problem is to see whether any basis exists for choosing, among all drugs and other chemicals to which man is exposed, those few that are most likely to present a genetic hazard, and to concentrate further research energies upon them.

My entire presentation here today may well be subjected to the criticism that it is premature, that it makes much out of little. Indeed, I hope such a criticism turns out to be valid. But it must be pointed out that we are quite properly concerned about genetic effects of radiation, despite the fact that there is as yet only slim evidence for such genetic (in contradistinction to somatic) effects in man. Yet we do not hesitate to extrapolate from a variety of other species and we assume that the chemical behavior of man's genetic apparatus is not wholly unique. It is true that there is even less evidence (i.e., none at all) for an effect of any chemical mutagen in man or, for that matter, in any mammalian organism, except for a very few experiments with alkylating agents in mice. However, it would seem improper to dismiss chemical mutagenesis in man merely because the data thus far are weaker than in the case of radiation. Experimental evidence leads to practical concern, but it is also true that concern often leads to experiments that yield new evidence.

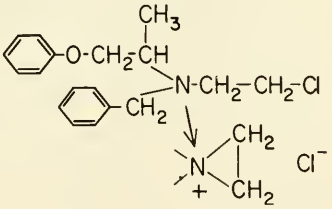
NITROGEN MUSTARD



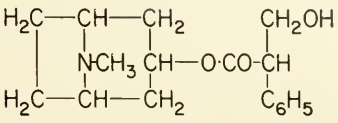
MORPHINE



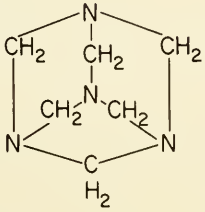
PHENOXYBENZAMINE



ATROPINE



METHENAMINE



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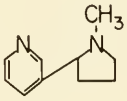
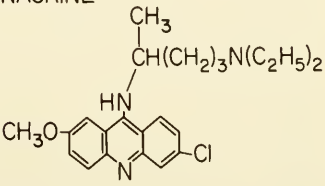


QUINACRINE



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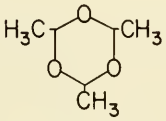

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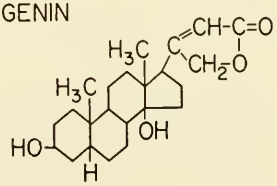

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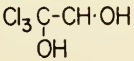
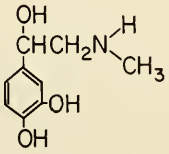


EPINEPHRINE



HYDROCORTISONE

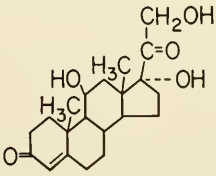


Figure 24. Some examples of compounds which are mutagenic or related to mutagenic compounds, and are also used as drugs.

If we consider the type of chemical mutagens that were discussed yesterday by Dr. Auerbach, we find that for almost every type of compound she discussed, which has been shown to be mutagenic or to cause chromosome abnormalties without actual demonstration of point mutations, there are closely related compounds in the world of drugs which are used with greater or lesser frequency in man. (Compounds discussed in this section are shown in Fig. 24.)

Take the most striking example—the alkylating agents. Compounds of this group are employed in the chemotherapy of certain kinds of cancer. An interesting relationship exists between carcinostatic, mutagenic, and (curiously) carcinogenic agents. Some substances have all three of these actions (4,12,29).

It is certainly possible that agents of this type may owe their mutagenic and anticancer effects to the same or similar action, for example, through interference with chromosome replication in the cancer cells, which are characterized by a rapid growth rate. It is clear, at any rate, that they do not uniquely attack cancer cells but owe their major toxicity to the production of detrimental changes in rapidly growing normal tissues, primarily the bone marrow and the epithelium of the intestinal tract, which undergo continuous rapid cell division.

Those who hold to a somatic mutation theory of the origin of cancer readily explain the similarities between carcinogenic and mutagenic agents on the basis that the carcinogens can induce a somatic mutation which leads to the development of a clone of cancerous cells.

A final aside on the question of teratogenesis, i.e., the induction of abnormalities in the developing fetus (31). A number of teratogens are also known to be mutagens, and it is conceivable that in some cases of teratogenesis (although this has never been proved), the developmental abnormality results from somatic mutation at a critical time in fetal development, so that particular organ systems are affected.

In general, when cancer is treated with alkylating agents or other compounds which are known to be mutagenic, the genetic hazard can be discounted because the type of patient who is receiving the treatment is generally beyond the reproductive age. However, there are some instances, such as in Hodgkin's disease, where the individual is often within the reproductive age. This suggests an area for investigation as to whether patients so treated and subsequently having children can be shown to have had genetic damage induced.

Here one knows that the alkylating agents do act upon the neoplastic cells, and there would seem to be no good reason to doubt they also act upon the germ cells.

Motulsky: A few years ago, I became interested in the problem of mutations induced by chemotherapy with alkylating agents in Hodgkin's disease and related disorders. I wrote to a number of people to get cases of this sort. In most instances, both male and females became sterile after the treatment.

Goldstein: Apart from their use in the treatment of cancer, alkylating agents may be used for an entirely different purpose. This, I think, is worth some discussion, because it is a case in which something could easily be done. There is a compound known as phenoxybenzamine which is a one-armed nitrogen mustard, and which, for reasons that nobody understands, is specifically effective in the body in blocking adrenergic receptors. For the diagnosis of certain types of hypertension, this drug has been used to lower the blood pressure by making the arteriolar receptors unresponsive to circulating sympathomimetic compounds. The very long duration of the adrenergic blockade is consistent with an alkylating mechanism. In a compound of this kind, there is a cyclizing to form an immonium ion which is thought to be the active alkylating agent. These active ionic intermediates can then be destroyed by hydrolysis in water or in serum, but at rates which vary greatly from compound to compound (53).

It is not known why this type of alkylating compound has its specific pharmacological effect, but on the basis of its alkylating properties it can be assumed until proved otherwise that such a drug is mutagenic. I think this should raise a serious question about the wisdom of its use especially since other drugs can achieve the same pharmacologic purpose.

Addendum: There has recently been reported (37) a new class of ganglionic blocking agents which would presumably be used in the treatment of hypertension. These are modified nitrogen mustards which take advantage of the good oral absorption of uncharged compounds. Once in the blood stream, the cyclic immonium ion is formed, conferring the positive charge required for effective ganglionic blockade. These proposed drugs, known as "pronium" compounds, could, I believe, be considered *a priori* to be mutagenic alkylating agents and hence genetic hazards to man. Since other types of compound are available for reduction of blood pressure, there would seem to be no good reason to risk the therapeutic employment of "pronium" derivatives.

It was mentioned yesterday that aldehydes have been shown to be mutagenic in a number of organisms, although the mechanism is not clear. They may act through the formation of a complex with peroxides. Certain aldehydes are in use as drugs.

A straightforward example is methenamine, which is a condensation product of ammonia and formaldehyde. This compound is a urinary

antiseptic. It is taken by mouth and absorbed well, and it ends in the bladder where, provided the urine is acidified, there is a release of ammonia and formaldehyde. Formaldehyde is clearly mutagenic in some systems. But we do not know the extent to which formaldehyde released in the bladder, or the smaller amounts released during the passage of methenamine through the blood, may have access to the germ cell. Equally unknown is whether the formaldehyde in the bladder may ever be carcinogenic.

Another compound of interest is paraldehyde, which is a trimer of acetaldehyde. It is a sedative, often used in manic alcoholics. It is injected intravenously in very considerable doses, and has an excellent sedative action. But its metabolism in the body is at least in part a breakdown of the trimer to release acetaldehyde.

Atwood: Why is it so nontoxic if it makes acetaldehyde?

Goldstein: The acetaldehyde levels are very low. Right now I am not concerned with quantities but only with pointing out that these drugs exist, and that they (or their metabolites) belong to certain classes that are known to be mutagenic. It may very well be, from the standpoint of practical mutagenesis, that the amounts involved are absolutely negligible.

Auerbach: Rapoport (49) found that acetaldehyde is a much weaker mutagen than formaldehyde.

Goldstein: It has not been adequately tested in other systems (18).

There is also chloral hydrate, which was, and in some places still is, a very popular hypnotic agent, so that a great many people have been exposed. This drug is a hydrated form of trichloracetaldehyde. It is a spindle poison and mutagen (5).

Phenols have been shown to have mutagenic effects, and a great many drugs, as you might expect, are phenols. I am not clear to what extent the phenol group in itself confers mutagenic action or whether only the simple phenols and cresols that have been investigated are mutagenic.

Examples of naturally occurring phenols are the catechols, such as epinephrine. Many complex drug molecules such as morphine contain a phenolic group, and—

Novick: Also, tyrosine.

Goldstein: Yes, tyrosine is a naturally occurring one. Now, a few words about nitrites. Nitrous acid is well established to be a potent mutagen. Sodium nitrite is still used as a preservative in certain kinds of food, particularly in meats, where it is accepted as safe on the basis of ordinary toxicity considerations. However, in the acid environment

of the stomach, where the pH is about 1 in normal individuals, it is obvious that nitrous acid will be formed. How much of that nitrous acid has access to the gonads, we do not know.* But one could easily argue, on the basis of general knowledge already at hand, that nitrites should be forbidden, since other preservatives are presumably available.

As drugs, nitrites are employed to improve the circulation in certain organs, particularly the heart, and sodium nitrite itself finds use as a long-acting dilator of the blood vessels. At the pH of the blood, 7.4, we may consider that the amount of nitrous acid will be negligible, but again, we must ask what is a negligible amount from the standpoint of mutagenic action.

Freese: Is it known how nitrite exerts its preservative effect?

Goldstein: I am not aware of it. It may be known.

Freese: I mean, could it be the very fact that it is mutagenic?

Lederberg: I think it's a heme poison.

Goldstein: It is a heme poison in the sense that it produces methemoglobinemia in man, in toxic amounts. It takes quite a bit to do this.

Magni: Is it for preserving the color of the meat?

Lederberg: I think it is primarily used as an antibacterial agent.

Magni: No, it is not bactericidal. *E. coli* can grow in the presence of 1 mol/liter of sodium nitrite in neutral media.

Goldstein: I think there is no convincing evidence that it is mutagenic. To continue with examples of different types, some drugs have been shown to be mutagenic or to produce chromosome damage without any specific functional groups being clearly involved. Atropine, for example, may be cited as representative of a class of complex molecules which finds wide use in medicine, which has been shown to have this effect, and in which one has no idea what the mechanisms of mutagenic action may be.

Motulsky: What are the systems in which they have been shown to be mutagenic?

Goldstein: It is generally in plant systems, where the criterion is the observation of chromosomes (18).

Zamenhof: Wasn't ethylene oxide recently suggested as an agent to sterilize canned food? That would also be a possible mutagen.

Goldstein: If there is a residual, yes, but that is unlikely.

Glass: Did you intend to suggest beta-propiolactone, which has been suggested for medical use recently?

Goldstein: No. Would you like to?

*Nor, again, whether the higher concentration in contact with the gastric mucosa might be carcinogenic through induction of somatic mutation.

Glass: I don't remember for what it was proposed to be used.

Lederberg: To kill viruses.

Glass: Yes, to kill hepatitis virus, I believe it was. This, of course, is known to be one of the most potent chromosome-breaking agents that has been worked with in plants, and our studies with tissue culture show that it breaks primate chromosomes just as readily as it breaks plant chromosomes.

Freese: It is very mutagenic in bacteria.

Auerbach: It also has a curious property which the ICI people found. It is one of the very few substances that are carcinogenic but not carcinostatic.

Atwood: That was to be added to bank blood to prevent the spread of serum hepatitis. There wouldn't be a residual if it were added.

Goldstein: I want to mention nicotine, and I will come back to it again. It has been shown to cause chromosome damage in plants (5), and, of course, there is wide exposure of the human population. We also have an example of an acridine-type compound which has had wide use in medicine—quinacrine (also known as atabrine), which was for long the mainstay of antimalarial therapy and prophylaxis. Some acridines are certainly mutagenic (5), but I am not aware of any evidence that this particular one is.

Finally, I should like to point out that many steroids are used medicinally (e.g., the digitalis group and the adrenal cortical steroids) and that some steroids are strong mitotic poisons (5). I shall discuss later a particular steroid which is proposed for very widespread use as an oral contraceptive agent.

I have not listed any of the purine analogues here because I expect to talk about caffeine and related compounds at length.

The purpose of this enumeration was not to make any special point, but only to indicate that compounds known to be mutagens or chromosome breakers, and closely related compounds, are used widely in medicine, and no one has yet given much attention to the question whether or not they may be genetic hazards.

Auerbach: May I raise a general question which I would like to have discussed, if somebody has evidence on it? On going through the literature, I got the impression that plant chromosomes have a much more easily disturbed structural stability than animal chromosomes. I don't know whether there is any evidence from tissue culture. Anoxia, too much oxygen, or too little oxygen, aging—all these conditions seem to produce chromosome breaks in plant cells.

Auerbach: In tissue culture?

Glass: In our tissue culture studies, the frequency of spontaneous breakage, from Bender's data (7,8), ran around 1 per cent of untreated cells with chromosome breaks, so that you could say that the ordinary methods of culturing certainly are producing a fairly high percentage of chromosome disruption, unless you want to suppose that this goes on *in vivo,* too. I don't really think there is any warrant for that.

Atwood: There is good evidence against it, in fact.

Glass: Yes. The agents in plants mostly have not been tried as yet in tissue culture. We are just beginning this kind of work.

Auerbach: What strikes me is that Drosophila chromosomes, at least those in the germ cells, require high doses for breakage. To get a moderate yield of translocations one uses at least 1000 *r*. For plants, this would be a very high dose. I think, though, that in tissue cultures, animal chromosomes can also be broken by low doses. Did not Puck find an effect with only a few *r?*

Glass: Yes. Bender finds that a dose of about 200 *r* would produce at least one chromosome break in every cell.

Auerbach: Drosophila sperm chromosomes are then not a typical representative of animal chromosomes.

Bearn: The Edinburgh group have recently shown significant changes in both chromosome number and structure following therapeutic X-ray therapy to patients with ankylosing spondylitis (58). Maximum changes occurred after three days of exposure and decreased rapidly thereafter. In other patients, well-marked changes were observed within 24 hours following a single dose of X-rays of 250 rads directed to the spine.

Goldstein: This is a discussion I hoped would come up, because so much of the evidence on "mutagenic activity" of the compounds I am talking about is based on chromosome breaks, and I found it very difficult to evaluate. Why should compounds which are mutagenic in the strict sense of the word be chromosome breakers at all? This is not clear to me. One is struck with the fact that a number of compounds, not alkylating agents, which produce what seem to be point mutations, are also chromosome breakers. If there is a similar mechanism here, then one has to take seriously the evidence based on plants. If there were a basis for throwing it out, we might feel happier about a number of drugs.

I would now like to make some general remarks about the extent of exposure of human populations to exogenous compounds. We must consider not only the potential genetic hazard of compounds that have recently come into pharmacological use. We must recognize that

the human race has been exposed to a changing chemical environment for a genetically significant period of time, i.e., for thousands of generations. The development of agriculture, for example, provided new kinds of foods as major items of diet which were not available before. The discovery of fermentation introduced a wholly new organic compound to which man was not previously exposed. Certain religious customs have created special exposures to exogenous substances, for example, the peyotl rites among some American Indians.

Medication goes back to the days of prehistory. Professor Dunlop in Edinburgh used to begin his course in therapeutics by saying that the main feature which distinguishes man from the lower animals is his desire to take medicine. Man has always sought in nature remedies for his physical and mental ills. Particularly prized have been psychic stimulants. For example, caffeine, being the main stimulant alkaloid of tea, was in use as a stimulant at least as long ago as 2500 B.C. in China. This is more than a hundred and fifty generations, a long time for man, and an absolute minimum estimate of the duration of exposure.

It follows from all this that we have to regard the human race as extremely heterogeneous, both in space and in time, with respect to exposure to exogenous compounds. Were some of these even weakly mutagenic, continuous exposure over a long period of time might have had some significant effect upon the human germ plasm.

Although some of these exposures have gone on for hundreds of generations and more, an extraordinary change has occurred in very recent times. The total exposure to exogenous compounds in our environment has increased explosively primarily as a result of developments in synthetic organic chemistry and its offshoot, the pharmaceutical industry. This remarkable state of affairs had its inception only in the middle of the last century, or not more than three or four generations ago. Thus all of us are exposed with regularity to compounds which did not even exist a short while ago, while the number and variety of these compounds and the extent of exposure continues to increase with great rapidity. If any of them had significant mutagenic activity some doubt would be cast upon the concept that we have attained a stable genetic equilibrium in man. Moreover, the question of "spontaneous" mutation in man must be viewed against the background of this exposure to compounds which might possibly be mutagenic. We heard something the other day about estimation of the spontaneous mutation rate in man. There is a considerable genetic

load due to spontaneous mutation, perhaps several mutations per gamete. What are the sources of the spontaneous mutation rate?

There is agreement that all sources of radiation, including cosmic radiation, natural radioactivity in our earthly background, the slight additions as a result of fallout in recent years, and also the effects of transmutation of naturally occurring C^{14} in DNA—that all these could account for less than 10 per cent of the estimated spontaneous rate in man (47, 56). Then 90 per cent must be attributed either to undefined instability of the genes or to chemical mutagenesis, caused by endogenous or exogenous substances. A major role of chemical mutagenesis in spontaneous mutation is suggested by Novick's (43) finding with *E. coli* that two-thirds of the spontaneous rate could be suppressed by an antimutagenic nucleoside.

It is at least within the realm of possibility that exogenous mutagens are making a major contribution to the present spontaneous rate in man, and have made a significant contribution to the existing genetic load of deleterious mutations. Finally, we must recognize that no mutation rate estimates in man have ever been made in the absence of exposure to exogenous substances, some at least of which are known to be mutagenic in other species.

I should like to go on now to a general feature of the mechanism of drug action which has certain implications for any possible genetic hazard of drugs. With the exception of a very few compounds (like the alkylating agents) drugs are remarkably unreactive in the ordinary chemical sense, but owe their biological effects to highly specific reversible combinations with functionally important tissue macromolecules ("receptors"). These drug-receptor interactions depend upon electrostatic bonding, hydrogen bonds, and above all Van der Waals' forces, which are so sensitive to small configurational differences between closely similar drug molecules. It is therefore unlikely that most drugs, no matter how biologically potent they may be, could directly attack and chemically alter the DNA or associated structures of the genetic apparatus.

We should distinguish here between two principal modes of chemical mutagenesis. On the one hand there is direct chemical modification of the gene, as in the deamination of nucleic bases by nitrous acid or the alkylation of guanine by diethylsulfate (see p. 101). Such alterations of the DNA can be effected even *in vitro,* so that active replication is by no means a prerequisite. On the other hand, genetic alterations can be induced by intervention in the replicative process, either through incorporation of abnormal base analogues or by otherwise

altering the mechanism of replication of chromosomes and their genes, or of their mitotic (or meiotic) separation. It is reasonable to suppose that most drugs, were they to cause mutations, would do so in this second way. This in turn suggests that during periods of rapid cell division in the germ line the hazards of this type of chemical mutagen should be especially great, and conversely, that during periods of dormancy in the germ line the risk should be diminished.

The main reason for trying to define periods of relative safety or hazard is that if such an analysis were valid in man it would dictate practical approaches to the reduction of genetic hazards of this type. Can we make any quantitative estimates of the relative importance of the various periods in human life? This is attempted in Figure 25, for the male. The diagram depicts schematically from left to right the period of about 30 years from zygote to zygote. Period 1 occupies the first 3 months of fetal life, during which the zygote gives rise to all the somatic cells as well as to an established line of primordial germ cells (primary spermatogonia). The actual migration of early germ cells into the gonadal areas and their rapid multiplication there occupies a fairly brief period at about the sixth to eighth week of fetal life. In the female, further differentiation to oocytes proceeds, followed (it is thought) by dormancy throughout childhood and thereafter until the final reduction divisions are completed, one ovum at a time, during reproductive life. In the male it is probable that stem cell divisions occur regularly (36) with degeneration of cells before they reach the spermatozoon stage, throughout childhood (Period 2). At puberty the regular maturation and shedding of sperm begins and continues throughout reproductive life (Period 3), during which stem cells continue to give rise, through unequal divisions, to sperm clones and to more stem cells. Period 4 covers the maturation of any given sperm clone.

What is the probability, P, that a given sperm will be mutant with respect to a particular gene? It is the product of the probability of mutation of that gene per cell cycle by the number of times it has been at risk in the replication process, i.e., the number of cell divisions in the lineage of that sperm all the way back to the zygote, or at least to the differentiation of germ cells in the young fetus.

Let p_0 be the probability that replication of a given gene in the presence of a standard concentration (e.g., 1 M) of mutagen will lead to one mutant progeny. I shall assume that for chemical mutagens, as for radiation, there is a concentration effect, and that this is linear. There is good evidence for a concentration effect in Novick's work

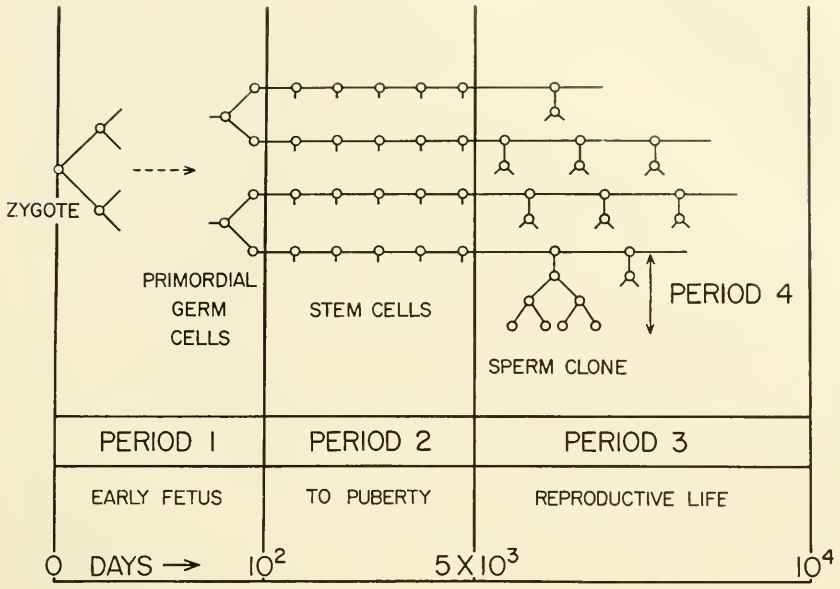


Figure 25. The germ line of man.

A schematic diagram showing the four periods of possible sensitivity to mutagenic agents in the human male. The average span of a human generation from zygote to zygote is taken to be 30 years (approximately 10^4 days).

with caffeine, but those data are certainly not enough to establish linearity. I shall assume it, and if anyone wants to introduce further evidence on this point, I should be glad to hear it.

Benzer: There are very good data on nitrous acid, by the Tübingen group, over a wide range of concentration (59).

Goldstein: But that would not fall into the category I am talking about, presumably, since you induce mutations *in vitro*. I shall assume, then, that there is a mutation probability for replication in 1 M mutagen and that the actual probability (p) is a definite fraction of this standard probability, determined by the concentration (c) that is actually present. Thus, $p = p_0 c$.

I am also going to assume that the probability of mutation per cell cycle is the same at all stages of development of the germ line and of the sperm. This may be entirely wrong, but if it is, corrections are relatively simple to introduce.

Let m be the total number of cell generations in the lineage of a given sperm. This is to be segmented according to the 4 periods depicted in Figure 25. Thus m_1 is the number of cell generations from zygote to establishment of the stem cell population; m_2 is the average number of divisions of a stem cell during childhood; m_3 has the same meaning from puberty through all of reproductive life; m_4 is the small number of cell generations within the sperm clone itself from the unequal stem cell division to the final spermatozoa. Let t be time, in days; g be mean generation time, in days; n be an estimate of the total number of genes in the human gamete.

After continuous lifelong exposure to a mutagen, the probability that a given sperm will be mutant with respect to a particular gene (which is the same thing as the frequency of such mutants in a sperm population) can be represented as the sum of four different terms,

$$P = \tfrac{1}{2}\ (m_1 + m_2 + m_3 + m_4)\ p.$$

Now I am going to assume that we can speak of an average mutation probability for all genes in man, so that by multiplying the above expression by the total number of genes (assumed to be 10^4) we can obtain an estimate of the probability that a sperm is mutant in any respect at all. It will also prove helpful to substitute for m_3 the equivalent expression t_3/g_3, where g_3 is the mean generation time (days) in the line of stem cells during reproductive life. We then obtain

$$P = \tfrac{1}{2}\ (m_1 + m_2 + t_3/g_3 + m_4)\ np.$$

The number of cell generations in the early establishment of the germ line in the fetus, m_1, is obviously a fixed quantity in man. I am going to estimate that 30 may be the correct datum here, although I would welcome correction based on actual data. This is a fairly important figure because it determines the relative importance of fetal life and later life with respect to mutagenic exposure. Dr. Atwood the other day brought this out and suggested that the fetal period might be equivalent to ten years of later exposure.

As for m_2, the ensuing period until puberty, this term and m_3 both probably have the value zero in the female. In the male, if we assume pubertal age 5×10^3 days and stem cell mitosis every 12 days in childhood as after puberty, we obtain approximately 400 stem cell generations as the value of m_2. In the subsequent term, g_3 will also be given the value 12 days, which is probably the period of the seminifer-

ous cycle in man. Finally, m_4 in man is a very small number, no greater than 4 or 5, and in woman evidently only 2.*

Atwood: The mutant frequency should be higher at the end if you have had clonal growth.

Goldstein: We disagree on this, as we did the other day. There is no formal difference between the growth here in the stem line and the growth here in the sperm clone. This whole thing is one large clone (see Fig. 2), and the probability that a cell anywhere in it is mutant is a function of the number of times it has been at risk.

Novick: Kim, would you think it were correct if the population size is very large? Wouldn't you think it is simply a question of population size that is important?

Atwood: I fail to understand you.

Novick: I meant population size in number of cells, either sperm or spermatogonia present. If the number of spermatogonia can be considered as very large, in fact, then he is correct. If the number of sperm is very small, then one has to use some nondeterministic expressions. I think this is what you were concerned about.

Atwood: It wasn't, but it should have been.

Goldstein: It is a basic point. It was at issue the other day and it seems to be at issue again. Let's see if we can agree about the analysis of an ordinary clone. How do you analyze the probability for an ordinary bacterial clone—what is the probability that a given member of the final population will be mutant? I am saying that the probability that this cell is mutant is equal to the probability of mutation per cell division times the number of cell divisions that have occurred in the history of that bacterium.

Neel: Instead of saying "per cell division," would you say "per cell cycle," to keep Dr. Zamenhof happy, and me, too? It is per cell cycle.

Goldstein: Yes, per cell cycle; all right.

What I am trying to ascertain is the relative importance of the

*The figures used here for m_1, m_2, g_3, and m_4 are the best I could obtain. C. P. LeBlond (personal communication) supplied estimates based on work with the rat and some suggestions as to the probable values of the several parameters in man. The statement that stem cells divide regularly throughout childhood is an extrapolation from findings of Clermont and Perey (15) in the rat. The estimate of 12 days as the mean generation time in the stem line of man is derived from LeBlond's suggestion that one might be justified in assuming that cycle duration in man is 10 to 12 days, since figures of this type have been observed in many species. However, he points out that direct evidence in man is lacking. Commenting on the difficulty of making reasonable guesses about man, he writes: "Our knowledge of the human testis is pitiful."

various periods in human life for genetic hazard due to the exposure to chemical mutagens. It is essential to establish, for example, whether the fetal period is so important in the establishment of mutation (as Dr. Atwood suggested the other day) that this would be a time for concentrated antimutagenic efforts. It is very easy to take special care to see that women in the first three months of pregnancy are not exposed to mutagenic agents. This is a great deal simpler than worrying about the exposure to all mutagenic agents throughout our lifetime. If, on the other hand, it turns out that this period in human life is not so important after all, but is soon outweighed by the accumulation of mutants in the germ line, it necessitates quite a different approach.

The only way to find this is to have some estimates of the numerical terms, of the four terms in this equation, which represent four distinct periods in human life.

Novick: But isn't your clone inclusion going to be determined by whether or not you are going to assume that mutations occur at a constant rate per generation or per unit of time?

Goldstein: Yes, and I have already made that assumption.

Novick: If you assume per generation, the early period is going to be very important. If you assume per unit of time, then all time will be equally important.

Goldstein: I think it won't turn out that way. Let's try to find what actually is known about this.

Neel: One point of clarification. Your m_4 term refers to the divisions of the meiotic cycle?

Goldstein: Yes.

Atwood: Two divisions.

Goldstein: From the stem cell to the final sperm.

Neel: Good. I didn't understand it that way. This is fixed, then, at only two divisions.

Lederberg: Not necessarily only two. This is the actual number of terminal divisions of a cell which will give only spermatocytes as progeny; that is, at a certain point, a stem cell will divide in such a way that all the subsequent progeny of that cell become sperm, rather than continuing to divide to maintain the stem line. That represents the number of divisions after a cell leaves the stem line. I think that is the best way to put it. It may be more than two; in fact, it probably is. There is a little earlier time of determination.

Neel: In any case, this is a small number.

Goldstein: Let me ask a specific question. Do we know anything for certain about the mean generation time in the stem line?

Russell: I think some kind of answer is available, but I don't have it with me.

Goldstein: Can we make any kind of guess? The guess that Dr. Atwood made the other day was 100 days.

Atwood: That's for bone marrow. It's probably not too different, though.

Russell: In the female mouse, all mitotic divisions are completed before birth, and this is almost certainly true in the human. At 72 hours after birth, in the mouse, the oocytes go into what is called the dictyate stage, and they stay in this one cytological state until a few hours before ovulation. In short, there is no cell division of any kind in the oocytes, or chromosomal divisions, throughout the large part of the life of a mouse. Yet we can induce mutations in this period.

Goldstein: But not necessarily by such substances as would act during replication cycles. You can induce mutations with radiation.

Russell: That is a very important point.

Goldstein: Yes, it is because, once we accept this point, it means that the risk of exposure to mutagens of that type can be ignored for the female, except during the fetal period of growth.

Lederberg: What you are saying, Dr. Russell, is that for the female mouse, the second and third terms don't apply because there is no stem line after birth, and the fourth term doesn't apply, either, so there would just be a constant term for this type of mutation. As Dr. Atwood also mentioned, there would be no age dependence. What about the male?

Atwood: The fourth term has a value of two generations in the female.

Lederberg: That's right. But this is in conflict.

Russell: I think you're trying to restrict things to the molecular level in a way which may be an oversimplification when the kind of things Dr. Auerbach was talking about yesterday can be paramount.

Lederberg: I think we're asking histometric questions. Is it possible to say how many divisions are involved in the production of the germ tissue up to puberty as a constant term, and then, what is the turnover rate of the germ line subsequent to puberty?

Russell: As I said, I think rough answers can be obtained.

Goldstein: Let's suppose the value 30 is correct for m_1, the cell generations leading to the establishment of the germ line. We can certainly agree that m_4 will be a very small number. Then supposing that the mean generation time in the germ line were 100 days, as Dr. Atwood suggested for bone marrow, it is clear that the age effect becomes important only at a certain point, namely, when m_2+m_3 reaches the

value, 34; in other words, at 3400 days, which is ten years. That brings us back to Dr. Atwood's statement that the events in the very early fetal period are about equivalent to ten years of later life.

However, if, as I have suggested, the mean generation time in the stem line were 12 days, the position is entirely different. In one year in the male, we have already reached the equivalent exposure that we have in the fetal period, and everything after that is a continuous age effect. It does make a difference in estimating the importance of the exposure during the adult life.

Novick: Or if it occurs at a constant rate per hour, which is the opposite extreme.

Goldstein: Now let us return to our equation and substitute into it our known and assumed values for the several terms. We then obtain

$$P = \tfrac{1}{2}\ (30+400+t/12+4\ )\ (10^4)\ p$$

in the male, and

$$P = \tfrac{1}{2}\ (30+0+0+2)\ (10^4)\ p$$

in the female.

If these terms are even approximately correct, it follows that in the male the contribution of events during fetal life is very small compared with later events. This is true even at puberty, and the significance of what happened during fetal life becomes even less by the usual reproductive age. Moreover, the contribution of the period during which a sperm clone develops from the stem line is entirely negligible. The equation predicts a strong age dependence for the frequency of mutant sperm. Even if the estimate of stem cell generations during childhood were greatly exaggerated, the contribution of the first postpubertal year would equal that of the fetal period and the same age dependence would subsequently be seen. In the female, on the other hand, the dominant contribution is from the fetal period, and no age effect whatsoever should be manifested unless, indeed, an unrecognized stem cell mechanism is operative in the female as in the male. It should perhaps be emphasized again that I am speaking only about mutagens whose action is upon the replication or division process.

There is a point that I wanted to bring up in connection with the question of the female oocytes remaining dormant for many, many years. One could ask the question whether, during this period, there is, in fact, turnover of DNA or some kind of continuing replication and degeneration of cells. There is a recent paper by Bennett and Skipper (9) demonstrating the conservation of DNA in the liver and brain

of the mouse. This is not specifically related to the germinal tissue, but one can at least see that what one thinks of as a little implausible can really happen.

They injected C^{14}-adenine into a pregnant mouse in order to label the DNA of the fetuses and then sampled the young mice after birth at various intervals up to one year, half of the life expectancy of the mouse. Throughout this time there was turnover of RNA but not of DNA; that is to say, there was growth, there was addition of new DNA in the liver, for example, but the label which had been introduced was entirely conserved. This would indicate not only that the cells formed in fetal life remained essentially intact, but there was also no significant turnover of their DNA, which would have been detected as a decrease in total radioactivity in those two organs. The same result was obtained in the brain, an organ that for a long time has been supposed not to undergo further mitosis and cell division after birth. In that sense it is comparable to the germ line of the female. These data therefore show the plausibility of a very long dormant period for the female germ cells and their DNA.

Let us return now to a concrete discussion about drugs. If one asked which kinds of chemical compounds have ever been shown to be mutagenic, and then examined each drug that might be similar to such a compound, the task would be almost hopeless. It seems to me more sensible to place primary emphasis upon the way drugs are actually used, the extent to which the population is exposed to various agents, keeping in mind the differing importance of the various periods of life for males and females. Such a classification of drugs has never been made because the issue we are dealing with here has not arisen before. Category I* would contain drugs to which people are exposed only occasionally and briefly, and which can therefore probably be dismissed from consideration as possible genetic hazards on that ground alone. Category II would contain drugs to which special groups of people may be exposed for long periods of time. Here mutagenic potentialities would definitely present a problem for the exposed individuals, but since large population groups are not involved, the genetic hazard to the race would be minimal. Finally, Category III, upon which I want to dwell, contains drugs and other chemicals to which a large fraction of the population is exposed for long periods of time, even for an entire lifetime.

Magni: Excuse me, but this classification is probably done on a logical basis. Could it be reviewed on another basis, that is, not the

* The long lists of drug names have been omitted here.

logical use of these drugs, but just the total amount produced in the world, divided by the world population, for every drug?

Goldstein: Actually, Dr. Magni, some drugs would have to appear in more than one category. Morphine would be in Category I, referring to its casual use—all of us have been exposed to it at one time or another in connection with a surgical procedure or something like that. However, for the special group of addicts it falls into Category II. Likewise, aspirin would appear in Category I because most people have taken an occasional one but it is not a long-term, continuous exposure. On the other hand, if you consider the world production, there's an awful lot of exposure to aspirin in the world population. So you do have to look at it in both ways. The risk to the individual and the risk to the germ plasm of the human race in many cases may be quite different matters. In a sense, therefore, the classifications are very arbitrary.

If there are drugs to which a large part of the world population is exposed occasionally, as in the case of aspirin, or drugs that some people are exposed to for long periods of time, such as atropine, to which people with peptic ulcer may be exposed for many years, are we obliged, or is it useful, to test them all to make sure they are not mutagens? This is something that may some day be raised as a practical matter as more interest develops in this area, and I think it is worth some discussion.

I am going to ask Dr. Novick if he would consider for us some of the problems that would arise in devising any kind of standard test of mutagenesis which could be run routinely, much as all drugs are tested for toxicity before they are released upon the population or put into the physician's hands.

Novick: I think that the moral is an obvious one—there is no such thing as a standard test that one could contrive for mutagenesis. Any evidence of mutagenesis in any organism is prima facie evidence for concern, and it would then depend upon the clinicians to discover if there is any cause for concern in man. If you find that caffeine is mutagenic in bacteria, then one ought to consider it a hazard for humans, *a priori,* until found otherwise.

Lederberg: From the somatic standpoint?

Novick: Both the somatic and germinal standpoints.

Atwood: Two things can now be done in man. If you want to see whether human chromosomes are broken by these agents, you could use the *in vitro* phytohemagglutinin-induced mitosis in the white blood cells, which occurs within 24 hours after drawing the blood. For some reason yet unknown, if phytohemagglutinin is added, the cells

divide (46). Chromosome breakage by various agents could be surveyed *in vitro* with this technique.

The second thing you could do is use an isotope dilution method in blood to find out whether any agent is radiomimetic. I don't say it tells you whether an agent is mutagenic, but at least it would tell you whether it behaves like radiation. Radiation, obviously, causes too large an increase in the minor populations to attribute just to specific locus mutagenesis, but some of the drugs that are mutagens might also show that large effect.

Auerbach: I should like to say that if any screening test for the effects of mutagens on man is to be used it has to be used on mammals. In addition to the method suggested by Dr. Atwood, I should like to mention another one, which is somewhat more laborious but has already given promising results. This method was first used by the Kleins (34) in Stockholm and by Dr. A. N. Mitchison (41) in Edinburgh. It consists of testing mouse tumor cells for mutations—perhaps I should call them genetical changes—at a histocompatibility locus in the mouse. The mice are used as test tubes in which populations of tumor cells are grown. Tumors that have been induced in hybrids between two inbred lines with different histocompatibility alleles do not grow in either parent strain unless one of the histocompatibility alleles has been lost. What this is due to one does not know; but when the loss is specific, that is, when the altered tumor cells take in one parent strain and not the other, we may presume that it is a genetical change—mutation, or deletion, or crossing over. Dr. Dhalival (20) used this method in Edinburgh and produced such changes by TEM and X-rays. He has now gone back to Malaya, where he will go on with the work. I think the main drawback of the method is that treating tumor cells *in vitro* would not produce mutations by just those mutagens in which Dr. Goldstein is most interested, namely, those that require replication for producing mutations. But I don't think it should be too difficult to devise a method by which the tumor cells could be treated under conditions that allow growth, for instance, by giving caffeine to mice in which the cells can grow, and then screening in noncompatible hosts.

Goldstein: Does Dr. Glass think that tissue culture of human cells might contribute to useful screening tests of any kind?

Glass: Yes, I do. I think that that would be a very natural development in the use of the technique. In fact, as I said a while ago, we have already begun this with some of the chemical mutagens, beta-propiolactone, and some of the diepoxides.

Freese: I propose that one should test with tissue cultures the induction of point mutations, because I think the mistake has been made in the past that, merely because one could more easily detect larger alterations of chromosomes, these mutations have been examined more carefully. I think that there may be dangerous mutagens that are given as drugs or used as food. These agents are chemically unreactive and therefore expected to produce only small changes of the genetic material.

Neel: Can we go back to the question that Dr. Goldstein raised, which really hasn't been answered? What mammalian or nonmammalian system or combination of systems would be the best screening device for the mutagenicity of this whole series of compounds? Isn't this actually your question?

Atwood: I think we have agreed that none would be entirely satisfactory. However, they might be suggestive.

Goldstein: There were really two aspects of the question. One was whether any screening system in nonhuman systems would be of any predictive use at all, and, secondly, whether a screening system based on human cells would be useful. If one could determine the relative mutagenicity of a series of drugs, then certain practical things could be done about it. I think we can assume that for most drugs the relative mutagenicity will be unrelated to the nature of the drug action. There is almost always a variety of drugs with similar actions, and which one is chosen now usually depends upon competitive promotional efforts, patent situations, tradition, cost, and so on.

To give you a specific example of this, there is a substance very closely related to caffeine, namely, trimethyl uric acid. In screening tests that showed up caffeine as a chromosome breaker on certain plant systems, this compound was not (32). We do not know whether trimethyl uric acid has a similar stimulant effect on the brain to that of caffeine. Suppose it does. Then one obviously would choose that drug which had the desired pharmacologic action, but did not have the mutagenic capability. There may be similar examples.

Novick: May I point out the danger of that kind of argument? I did test tetramethyl uric acid, and I did find it was nearly as mutagenic as caffeine; in fact, it was worse, because the effect of caffeine can be counteracted by adenosine or by growth under anaerobic conditions, while that of tetramethyl uric acid is not counteracted very much under these conditions. I think the only conclusion, to answer your earlier question, is to use a wider range of screening techniques.

All we can do is form a basis for suspicion, so that people who finally

go to tissue culture techniques, or ultimately the clinician, have some idea what to look for.

Neel: We're with you, Dr. Novick. All you can do is form a basis for suspicion. From the knowledge around this table of the test systems available, which are the best ones for formulating this basis for suspicion?

Novick: Dr. Auerbach pointed out, if not yesterday, at earlier talks I have heard her give, that, clearly, one should demonstrate this is a genetic change and should have a system where one can demonstrate that the effect is indeed a genetic one.

Atwood: I would like to augment your remarks about caffeine. The mutagenicity of caffeine, I think, was first discovered in Ophiostoma by Kihlman.

Auerbach: Yes.

Atwood: I tried it in Neurospora under conditions that I thought were the best; that is, they assured detection of mutants at any locus because I used the recessive lethal method. It is not mutagenic by this criterion at all, despite the fact that it was used in growing Neurospora in a race tube, at a concentration that reduced the growth rate to two-thirds of the normal value, and it even created a peculiar morphology of the thing as it grew. Yet, at the end, no mutants had accumulated whatever.

Novick: The same thing is true with bacteriophage.

Lederberg: I think you're putting the cart before the horse in asking this question about drugs. In so far as we have grave suspicions concerning an agent which is very widely used, namely, caffeine, I don't see how we can possibly want to distract ourselves by very serious consideration of a wide variety of other pharmacological agents until we dispose of this one.

Also, I don't know that there is ever any technique that is going to tell us about the mutagenic effect of any chemical as applied to the human population, if we're going to be making a variety of extrapolations from a variety of sources. If we were all agreed with respect to the mutagenicity of a given compound in Neurospora, *E. coli,* and Drosophila, I think that we would consider it very hazardous to assume it was not mutagenic in man. If we do find these discrepancies, we can ask the question, on which side of the fence does man happen to fall?

I think, as a preliminary screening test for the likelihood of the very general effect of a compound, the accumulation of bacterial mutants in the chemostat is unquestionably the most satisfactory and most

precise method there is for general purposes, wherever it could apply. We will want to try to look for point mutations in mammalian cells and cell cultures. Unfortunately, there have been very grave technical difficulties connected with this. I think there are many people working in this field and interested in mutation who seem to forget that we are dealing with a diploid tissue and, in some cases, a hyperploid tissue. Until we get around this particular barrier, we're not going to get very far with point mutations. We can look for dominant mutants, and there are some tricks we can do.

Atwood: The scarcity of markers is a point.

Auerbach: What about resistance mutations?

Lederburg: Drug resistance in bacteria, for the most part, has proved to be a recessive mutation. Frequently, drug resistance has been found in mammalian tissues. It is often associated with changes in karyotype, and I can think of no simpler explanation of this than the fact that it would require a chromosomal reorganization to unmask a recessive mutation in a heterozygous condition in mammalian cell culture, so we have here an extremely complex situation which is not going to be easily solved.

Either we take pains to do genetically well-defined material in cell culture, and that means mouse and not man, or we should take pains, I think, to try at least one small trick, that is, to use male rather than female cells, and hope to get sex-linked recessives which will at least expose part of the genome for observation. Alternatively, we might spend some effort in trying to find a haploid tissue that could be cultivated. This may or may not be a reasonable proposal. There have been reports that the syncytial trophoblast in haploids might be used. There may be other tricks we can use for getting this out. We may be able to use monosomic tissues which are now beginning to become available in man, and we can look for these in other experimental organisms as a way of exposing at least single chromosomes in a haploid state, to the point where we can look for point mutations in them. But as long as we use diploid tissue, the prospects are very remote indeed.

Glass: I want to differ with that point of view in one respect at least, although in general I agree fully. I believe there is an additional possibility that makes the outlook not quite so difficult, and that is the possibility of using human tissue cultures from individuals who are known to be heterozygous for recessive mutations.

Atwood: Provided it is a marker that can be used at the cell level.

Glass: That is the problem—to find the marker that can be detected in individual cells.

Stern: How could you eliminate the possibility that homozygosity would be the result not of mutation but of somatic crossing over or a similar process?

Atwood: That must be almost as rare as some of the mutations.

Stern: Is that known?

Atwood: I don't know, but at least we have evidence that it must be very rare in the blood cells.

Auerbach: I should like to say something about the possibility of scoring sex-linked lethals in mice. Dr. Falconer and I worked out a system for doing this and we have applied it to irradiated mice for two years, but the result was disappointing. I had calculated that if the lethal frequency in the screened section of the X chromosome was the same as the average mutation frequency found by the Russells, then we might obtain 2 per cent or more of lethals. In fact, we obtained none in several hundred chromosomes. So it seems that the section of the genome tested carries too few loci that can mutate to a lethal, and we abandoned the test. Carter in Harwell had a similarly disappointing result when he tested for autosomal lethals by a method designed by Haldane. On the other hand, Klein's system of treating tumor cells did give what appeared to be mutations both in his experiments and in those carried out in Edinburgh.

Atwood: Klein's system has the advantage of very powerful screening, comparable to the kind of screening we can do in bacteria, but could the induced compatibility be caused by chromosome loss?

Auerbach: Dhalival did a few cytological observations and as far as I remember he did get aneuploidy.

Lederberg: I saw him a few weeks ago, and it looks as though it is a crossing over, at least based on the observations. Where two components can be recognized from two individuals who are doubly heterozygous, so to speak, A and B, one gets out two types; one gets out the type where both of these factors become pure homozygous evidently, and types where one of these factors becomes homozygous and not the other. This would be compatible with crossing over, and it is a way of placing the relative order of the markers for the centromere. The technical possibilities of verifying this exist, because there are additional components within the H-2 region, and if one can obtain a seriation of these components by their pattern of homozygosity in these evident somatic segregants that agrees with the mapping that has already been conducted, I think it would pretty well establish the result. But at the moment there is only the bias that they get one type

of single component, nonheteroallelism, but not the other, which would make one suspect that it were mitotic crossing over.

Glass: May I take just two minutes to describe still another difficulty in this problem that has not been mentioned and that I think is rather instructive?

In the attempted use of markers in tissue cultures, we tried to use heterozygous albino rats and homozygous albino rats to provide two tissues derived from the pigmented layer of the iris of the eye usable for testing mutation in either direction, forward or reverse, at the albino locus.

The culture of pigmented cells proved unsuitable after a time because these cells, which were derived from the heterozygous animal, spontaneously lost their capacity to produce pigment after being kept in culture for a few weeks, so the percentage of nonpigmented cells would exceed 15 per cent, and you could not detect any mutations occurring at low frequencies in a culture like that. The albino strain—

Magni: Excuse me, but were these cultivated in the presence of mouse serum?

Glass: No, I think they were cultivated in human serum. I would have to check that to be quite sure. Mouse serum is a little difficult to get in quantity.

Stern: Just one question. Were homozygous pigmented tissues also studied? Did they lose their pigmentation capacity, in tissue cultures?

Glass: We started out with such cultures, but because it seemed impractical to look for mutations there, for the very reason that Dr. Lederberg suggested, we didn't press this; we didn't try to keep them going very long, and so I'm not sure.

Magni: I just want to mention here one experiment which has been done by Dr. De Carli in our department. He has been able to keep the antigenic property—that was group A, if I remember correctly—of a human sample for eight months, cultivating the culture in the presence of the homologous serum. If he shifted to another type of serum, the antigenic property was lost in two or three generations. This is just one example, but it could be taken into consideration.

Glass: My comment on the other strain of cells is the more significant one, I think. The albino cells were very constant in culture and never showed any signs of pigment at all, although they were subjected to a number of different treatments such as ultraviolet radiation, X-ray, and so on. No cells that had regained the ability to produce pigment were ever found. However, after studying these for a considerable period of time, Kodani and I became convinced that these

were not nonpigmented melanoblasts or melanocytes, but that since in the original explant of the tissue it is impossible to separate the melanoblastic cells from connective tissue elements also present in the iris, what you get is a replacement of melanoblasts by other cells which are of a different type, and never able to produce pigment in the first place—for example, fibroblastic types of cells.

Atwood: Do you think this is what happened in the heterozygous cultures that gave you the large numbers of unpigmented?

Glass: No, in that case, when the nonpigmented cells were examined, they were definitely of a melanoblastic type.

Atwood: What Dr. Stern implied by his question was how do you know those weren't the mutants?

Auerbach: Yes. The crucial comparison would then be with homozygous pigmented ones, to show that the white cells in your experiments were mutants.

Glass: These were untreated cells.

Auerbach: Yes, but the homozygous ones then should never, or hardly ever, give these white melanoblasts.

Glass: I think they did, but I don't recall the data.

Lederberg: Are you attaching some significance to this difference between the survival of the melanocytes or the presumptive melanocytes, as between the heterozygous and the homozygous, or do you think it is too big a chance variation of the experiment? When you say in the heterozygous tissues you maintain the melanocytes, in the homozygous explants, you did not maintain the homozygous melanocytes, are you attributing some significance to that? Do you think those cells are less able to compete with the connective tissue elements?

Glass: It may even be that in the iris of the albino the nonpigmented melanocytes do not exist any more.

Lederberg: There are two types of mutants in this respect, aren't there? I don't know what the situation is right here, but certainly in the mouse, in one case, the melanocyte does not exist; in the other case, you have an extreme dilution. There are cells which do not have pigment.

Glass: Yes. It would be possible to use a different allele, perhaps, and get it.

Neel: I think those of us who have been involved with the problem of the genetic effects of radiation in man know how quickly a concern can gain momentum in this country, with the result that one finds oneself confronted with questions from individuals and institutions who have policies to determine. I don't believe it is at all a wild con-

jecture that when the potentialities of chemical mutagenesis become known, within a few years we will be facing the same kinds of questions that have arisen regarding the radiation genetics of man.

I see very little likelihood that this problem can be satisfactorily studied in intact human populations. There are a few circumstances where one group of human beings has been irradiated and suitable controls exist. However, I find it difficult to visualize a satisfactory set of observations on intact populations involving caffeine. It is going to be difficult to set up one group of 300,000 drinking caffeine and a strictly comparable group of 300,000 who do not drink caffeine.

It looks to me, then, as if we are either forced to tissue culture techniques or else we are going to be guided for a long time by the results of screening tests on appropriate organisms. With respect to the tissue culture approach, we have already mentioned the many problems here. While the breakthrough may come at any time, it might be ten or fifteen years before all these problems are ironed out, so that I would guess that if public concern over these problems does arise, it will be the findings in microorganisms, Drosophila, and, conceivably, the mouse that are going to be guiding us for the next ten years. The question of the best combination of organisms to be used in forming an opinion will be raised again and again.

Goldstein: I would like to comment on some of these questions that have been raised, particularly on something that Dr. Lederberg said before. I don't want by any means to minimize the importance of caffeine and I will soon summarize the evidence that suggests that caffeine is the most important single compound for study. But the reason I raised the question of screening was, as our Chairman just suggested, that it is really quite accidental that caffeine came to our attention as a mutagen. Among the very large number of compounds to which human beings are exposed, both pharmacologically and otherwise, it is conceivable, although it is a nightmare, that there may be some which are really potent mutagens, about which one would want to do something. After all, caffeine seems to be a rather weak mutagen when you consider all the systems in which it has been tested. That is the reason I raised the question of screening, and I'm sure it is not going to be settled here, but this will be of concern, and I think that some approach will have to be made to it.

Let us now return to a brief consideration of Category III. I have tried to include here (Table 3) all chemicals, both drugs and substances in the environment, to which large parts of the population are exposed for long periods of time, regardless of whether or not anything is known about their mutagenicity.

TABLE 3

Category III: Drugs and other chemicals to which a large part of the population is exposed for long periods of time.

Ethyl alcohol.
Nicotine.
Water additives: chlorine, fluoride.
Foods and food additives.
Insecticides.
Industrial air pollutants.
Vitamins and trace metals.
Contraceptive jellies and creams.
Oral contraceptive: norethynodrel.
Caffeine and theobromine.

The first compound in this category is ethyl alcohol. At 0.5 M in the *Allium* test it produces abnormality in 50 per cent of all metaphase figures (6). Ethyl alcohol is a drug to which the human race has long been exposed, and to which individuals are exposed at irregular intervals throughout adult life. The concentrations established in the body are considerable. Very minor intoxicating effects occur at a blood level of about 1 mg/ml (0.02 M). The lethal concentration is around 5 mg/ml. Chronic exposure will differ significantly between wine- (or beer-) drinking countries and others. Ethyl alcohol is metabolized through acetaldehyde, and whenever ethyl alcohol is present there is also a small but measurable level of acetaldehyde. This raises again the question of mutagenicity of aldehydes. A recent report (40) indicates that in mammals, including man, ethyl alcohol is present as a product of normal metabolism at about 1 mM. This endogenous level is so low compared with exposure levels in drinkers, it can probably be neglected.

Nicotine is a drug to which there is very widespread exposure. There are figures, of course, on the number of cigarette smokers and the amount of nicotine that is absorbed. I think you probably all know that the total amount of nicotine contained in a cigarette is in the lethal range. It was once said that nicotine is so toxic that one drop on the tip of a cat's tail would kill a man.

Nicotine is usually absorbed in the amount of approximately 1 mg per cigarette, but sometimes much more, depending on the way a person smokes and whether he inhales deeply (54). There is certainly a circulating blood level of nicotine during smoking, and, in fact, if there were not a circulating blood level whereby nicotine reaches the brain, people who smoke for psychic pleasure would not smoke.

There was an interesting report in 1946 (57) that is worth mentioning. It was done by a pharmacologist who was interested in toxic effects of nicotine. He gave rats large amounts of nicotine in order to examine any direct effect on fertility. A large number of pairs of rats were used and the number of litters and the litter size were measured. Nicotine did, indeed, lower fertility in these rats. But the interesting thing was that he also kept the progeny and set them up in mating pairs. He found that in the progeny of parents who had been exposed to nicotine there was a statistically significant reduction in litter number and litter size even though these animals had not been exposed to nicotine during their own adult lives. Unfortunately, since the investigators were not looking for genetic effects in the first place, they failed to take certain precautions, for example, against the absorption of nicotine from milk during the weaning period. Moreover, the F_1 animals were certainly exposed *in utero*.

Spuhler: But there would be an independent effect on fertility?

Goldstein: There was an effect of treatment. There were adequate control groups of nicotine-treated parents and nonnicotine-treated parents, and nicotine-treated and nonnicotine-treated offspring. I think the design was adequate.

Auerbach: But they had not treated males and mated them to untreated females?

Goldstein: No, that's right. That would have been interesting to do. Because of the widespread exposure to nicotine in the human population, it probably would be worthwhile to devote more attention to potential mutagenic effects of nicotine.

There are in cigarettes, also, substances which are known to be carcinogens, and which have been the subject of much discussion in recent years; benzpyrene is an example. On the general grounds that many carcinogens are mutagens, these constituents of cigarettes also deserve investigation.

Certain additives to water supplies are worthy of mention, although I think it very unlikely they could be significant as mutagens. Water supplies are widely chlorinated, at a level of 0.2 to 0.4 mg/l. The absorption of this chlorine in any significant amount can be discounted because it reacts so avidly with organic material within the intestinal tract. Mutagenesis by chlorine is an open question but, like some other oxidizing agents, it might well have mutagenic actions in an isolated system of cells. But this is a negligible hazard in human populations.

Magni: It would be possible, anyway, to rule out this with a careful

comparison of European and American populations. In Europe, no chlorine is added to the drinking water.

Goldstein: Not so easy, I am afraid, because there are so many other difficulties in obtaining comparable groups.

Fluoride is also added to water supplies now, at a level of approximately 1 mg/l. This is a level that already exists in many natural water supplies, so some human populations have been exposed throughout the history of the race. The absorption of fluoride is excellent, but its mutagenicity is unknown.

In the realm of foods and food additives one could spend all day (and I don't propose to do so) talking about the numerous types of chemical compounds to which we are exposed. An example of a food mutagen is mustard seed, which contains the mutagenic allyl isothiocyanate (2). Garlic extracts produce chromosome changes in onion roots (18)! It is conceivable, as I mentioned at the start, that food habits in certain parts of the world may involve the ingestion of foods containing mutagens in significant amounts, but no data are available about this.

The addition of substances to food is another matter. Many types of compounds are added to food for various reasons—for buffering, coloring, preservation, sweetening or emulsifying, flavoring, antibiotics for preservation of meat and poultry, hormones that have been used to fatten the meat animal, and so on (10). Among all these one finds some compounds which have been shown to be mutagenic under some conditions, or are closely related to others that are mutagenic. But, on the whole, the amounts added are extremely small. In the case of the coal tar dyes, increasing evidence of carcinogenicity has led to their practical outlaw in foods. Nitrite is a distinct hazard already discussed.

Insecticides are used in the treatment of crops and fruits. The Food and Drug Administration tolerance limit is zero for residual insecticide, so that present intake may be disregarded, at least in this country. On the other hand, an insecticide like DDT is widely absorbed into the fatty tissues of meat animals and is present in the milk of dairy cattle exposed to DDT. As a matter of fact, autopsies on human subjects have shown that we all have measurable amounts of DDT in our depot fat. Thus exposure to this insecticide is quite general. But nothing is known about its mutagenicity.

Aminotriazol, the compound that figured in the 1959 cranberry scare, was shown to have carcinogenic activity, but its mutagenicity is unknown.

There is a group of industrial air pollutants that should be men-

tioned. Carbon monoxide is a widespread product of our urban civilization. You might suppose general exposure to be negligible, but, surprisingly, studies on the blood of individuals living in congested city areas, with a large amount of traffic, have shown that there is a constant carbon monoxide level, measurable by the carbon monoxide-hemoglobin level of the blood.

However, even large amounts of carbon monoxide are so tightly bound to hemoglobin that there is essentially no free carbon monoxide available for penetration into tissues. A report in the literature (38) tells of a carbon monoxide suicide of a pregnant woman at term. Carbon monoxide analysis was carried out on the fetal blood as well as the maternal blood, and there was absolutely none detectable in the former, despite the fact that the woman had enough carbon monoxide to kill her. So I think carbon monoxide can be dismissed from consideration.

On the other hand, ozone and other oxidants which are components of smog may well be mutagenic by analogy to other strong oxidizing agents such as peroxides that are known to be mutagenic. The absorption of ozone and similar compounds and their fate in the body and their mutagenicity in animal material are largely unknown, but this is a potential hazard in that large numbers of people (at least in some urban areas) are exposed to these atmospheric pollutants.

Vitamins and trace metals require some consideration. You would suppose that since these compounds are present naturally in the body, they could be dismissed. But, unfortunately, the one class of drugs with which people are most overmedicated is the vitamins, and the trace metals which are added to vitamin preparations. There is an enormous and wholly unwarranted across-the-counter sale of these substances, which are ingested in great excess over the natural requirement. If excessive amounts were mutagenic, we would have a real problem here.

Particularly interesting is the practice of overmedicating with B vitamins and ascorbic acid during pregnancy, when there is no evidence of vitamin deficiency, but simply the idea that large amounts of vitamin are probably healthful. Early pregnancy, as I have pointed out, may well be a period of fetal sensitivity to mutagens. Dr. Zamenhof will shortly present some data on mutagenic effects of ascorbic acid. These vitamin preparations, which are multimixtures, have trace elements of all kinds added to them fairly randomly. Iron salts are very common. I have seen preparations that include manganese, about whose mutagenic capabilities we have already heard. This is a wide-

open field for the manufacturers, since advertising and sales promotion can be aimed right at the general public. But since most use of vitamins is superfluous, even slight evidence of mutagenicity should cast doubt upon their continued indiscriminate use.

Contraceptive jellies and creams have to be considered, because although the exposure is intermittent, it is widespread. A very large number of compounds are used for direct contact contraception of this kind. If the contraception is effective, there is no immediate genetic hazard, but, as you know, the incidence of failure in contraception is considerable. I wonder about a sperm which has managed to get through this deleterious environment and fertilize an egg. Do we have something here analogous to the elegant little technique in Drosophila of causing mutations with vaginal douches and sperm baths? The kinds of compounds used for human contraception are principally detergents (both anionic and cationic), a variety of organic acids, phenyl mercuric compounds, and quinolines (17). Such compounds could possibly be tested under comparable conditions for mutagenic effect.

In the case of phenyl mercuric compounds, small amounts may be absorbed from the vaginal site into the general circulation.

An effective oral contraceptive has now been introduced for general use, approved by the Food and Drug Administration on the basis of its nontoxicity, and already administered to many thousands of women. Naturally, no thought has been given to potential mutagenicity. This is a progesterone-like compound known as norethynodrel, and by the trade name Enovid (48). Its structure is shown in Figure 26, where it is compared with the natural hormones progesterone and estradiol. There is no methyl group in the 19 position, where progesterone does have a methyl group, so this is termed a 19-norprogestational compound.

Norethynodrel acts by suppressing ovulation, much as progesterone itself ordinarily does during pregnancy. It is administered at 10 mg daily from the fifth to the twenty-fifth menstrual day; in other words, women are exposed almost continuously to this particular steroid.

Since some steroids at least have been shown to be mitotic poisons in plant cells (5), the effects of this particular one upon the genetic apparatus should be studied. Certainly we can anticipate that this compound, or others like it, will eventually be used on a nearly universal scale. On the other hand, for reasons suggested earlier, chemical mutagens (at least those acting exclusively on dividing cells) may present no great hazard to adult women.

Finally, in Category III, a very special position is occupied by

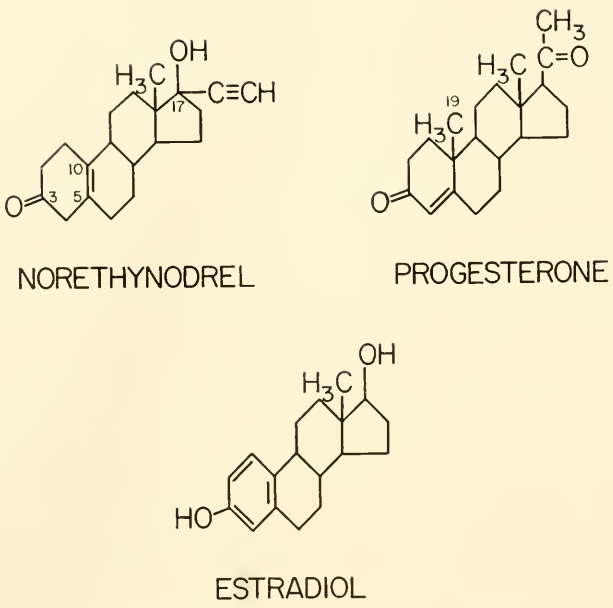


Figure 26. Structures of oral contraceptive agent and related hormones.

caffeine, and I shall dwell on it at length. The special position of caffeine rests on three facts: first, that it is certainly a mutagen (1, 19, 25, 26, 33, 43, 62); second, that people who are exposed to caffeine are exposed continuously, every day of their adult lives; and third, that a very large part of the population of the world is exposed, both in coffee-drinking countries like the United States, and in tea-drinking areas like Great Britain or the Far East. There has been some confusion about the caffeine content of tea, which I think should be cleared up. Caffeine is the main alkaloidal constituent of tea. People who drink tea for its mild psychic stimulation obtain that effect from the caffeine it contains. The amount of caffeine in a very strong cup of tea is roughly comparable with that in an ordinary cup of coffee. If people drink very weak tea, as in the Far East, the intake would have to be much larger to get the same effect, but the total amount drunk in those cases probably is much larger. So I think it would be fair to say that coffee and tea are approximately equivalent as sources of caffeine exposure.

Caffeine is depicted in Figure 27, together with the natural nucleic

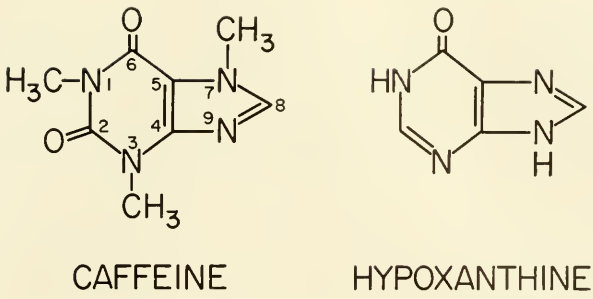


Figure 27. Structure of caffeine and hypoxanthine.
Caffeine = 1,3,7-trimethylxanthine; theobromine = 3,7-dimethylxanthine; theophylline = 1,3-dimethylxanthine. Hypoxanthine = 6-oxypurine; adenine = 6-aminopurine; guanine = 2-amino-6-oxypurine. Uric acid = 2,6,8-trioxypurine.

acid precursor, hypoxanthine. Theophylline is present in very small amounts in tea but is used medicinally for its diuretic and cardiovascular effects. Theobromine is a major alkaloid of cacao; a cup of chocolate contains about 250 mg. Coffee beans contain about 1 per cent caffeine, and also chlorogenic acid (a steroid), furfuryl alcohol, valeric acid, phenol, pyridine, and various volatile oils. An average cup of coffee contains about 130 mg caffeine. Tea leaves contain 2 to 3 per cent caffeine, cola seeds 1 to 3 per cent, and a glass of cola drink usually contains about 30 mg.

Atwood: Salicylates ought to be in your Category III.

Goldstein: I don't know. It's a matter of judgment as to where you put them. I think, if we asked in this room, "What was your caffeine exposure within the last week?" everybody would raise his hand and it would turn out to be considerable. But if we asked what the salicylate exposure was, I think that you would get a very small response. There is an important difference in degree of exposure.

Zamenhof: Dr. Goldstein mentioned ascorbic acid, and I want to say a few words about the mutagenic action of this vitamin. It was first reported by McCarthy (39) and then confirmed by us (61) that even in very small doses, ascorbic acid inactivates transforming principle. In our hands, a concentration of 10^{-4} M was sufficient for this effect, and, since agents which in very small doses inactivate transforming principle are also potential mutagens, it is of interest to investigate ascorbic acid from this point of view. Clark, in 1955, in the *Proceedings*

of the Oklahoma Academy of Sciences (14), had also reported that ascorbic acid was mutagenic in micrococcus (twelve times in the streptomycin marker and up to 300 times in the penicillin marker).

Ascorbic acid by itself is not a very important or very interesting mutagenic agent. It is auto-oxidizing and, while doing so, may produce some free radicals. Its action may be similar to that of ferrous iron. But what is interesting is the ubiquity of ascorbic acid. The presence of ascorbic acid in some cells has been reported, and although it has not been proved that ascorbic acid ever comes in contact with the DNA of these cells, it is possible that their life is impossible without ascorbic acid.

Using the phrase of Dr. Dobzhansky, that mutations are the only known raw materials of evolution, we may say that here we have a built-in evolution, that is, evolution inseparable from the cell.

This is merely to start a possible discussion on the very important subject of *normal* intracellular chemical mutagens, which act in addition to mutagens provided from the outside.

There are practically no publications on this subject except for Novick and Szilard's findings that adenine, which may be a normal component of the cell, is mutagenic (44).

After Dr. Goldstein's presentation, it came to my mind that glyceraldehyde, which is a normal component of the normal glycolytic cycle, may also be mutagenic if acetaldehyde is. Of course, the efficiency of aldehyde as a mutagenic agent will probably decrease as the chain length increases, but the glyceraldehyde is always with us and we have to take it into consideration. Perhaps, someone in the audience may suggest some other such substances of potential significance, which may contribute to the total so-called spontaneous mutation rate.

Goldstein: One class of drugs that is widely used is the barbiturates, which are pyrimidine analogues. We did some experiments recently, using T4-resistance in *E. coli* B to see if it was mutagenic, and Dr. Novick tells me that he also had done a similar experiment. Our experiment was with pentobarbital, one of the most frequently used hypnotic agents, at concentrations of 150 mg/l, which is the same as caffeine in Dr. Novick's experiments. This was not mutagenic at all under the same conditions in which caffeine was mutagenic. Incidentally, in our system caffeine was not nearly so mutagenic as in Novick's T5-resistance system. It increased the frequency of spontaneous mutants only some two to threefold.

Novick: One very important thing here, if I may interject, is the technique used to measure mutation rate. Before I would take such

numbers seriously, I would want to know precisely how the measurements were made.

Goldstein: These experiments were not done in a chemostat but under controlled conditions, in minimal medium, with the addition of mutagenic compounds at comparable—

Novick: Did you measure the mutation with what is called the Benzer mutation index?

Goldstein: Yes, or what I called P, which is the same thing. I am talking about mutant frequency at the end of exponential growth, where both the treated and the control cultures have reached the same total growth from the same small inoculum, and therefore have been at risk the same number of times.

Novick: It is difficult to speak of rate without taking into account selective effects.

Goldstein: I would like to continue my discussion of caffeine now. First, I shall consider the exposure of the population to caffeine. In order to make any estimate of potential mutagenic hazard, we have to know what actual concentrations of caffeine people are exposed to, and, more specifically, to what concentrations and for how long the gonads are exposed.

I mentioned before that the amount of caffeine contained in what we might call an average cup of coffee is between 130 and 150 mg. This, obviously, depends on the strength of the coffee and the person's individual taste. A strong cup of tea contains about the same. Cola drinks contain only a small amount of caffeine compared with coffee and tea.

There are two ways to get at the mean caffeine levels. One is by doing what Dr. Magni suggested before; that is, taking the total consumption derived from the sales of coffee, tea, and cola drinks, dividing by the total population to obtain a mean daily intake, and then, using our knowledge of the absorption and blood levels established from a given intake, calculate the mean concentration in the body.

The other way is to get evidence on the average number of cups of coffee or tea that people drink and then make the same kind of calculation. Both these lead to approximately the same result. In Table 4 are figures on the average coffee, tea, and cocoa consumption in this country for several periods. I don't have data for the war years themselves, but it is well known that the consumption of coffee goes up during periods of national stress. The major consumption of caffeine in the United States is in the form of coffee, as you can see, but

TABLE 4

Consumption of coffee, tea, and cocoa in the United States.

	1935-39	1947-49	1957
Coffee	14.0	18.2	15.8
Tea	0.67	0.58	0.61
Cocoa beans	4.4	4.1	4.1

Data are average pounds per year per capita. (World Almanac 1960.)

the picture would be very different in Great Britain, China, or Japan. Cocoa bean consumption is largely in the form of chocolate rather than beverages.

Next we have to know something about the absorption of caffeine and its distribution in the body.

Stern: May I just ask one unimportant question? Since you brought up the variation in amount of coffee per person, is this for the whole population or per adult person?

Goldstein: This is for the whole population.

Stern: There must be great variation in the number of children under 10 in the afterwar period as compared at least to 1935-39, I think.

Goldstein: That may well level it out. I have a calculation on the 1957 consumption, corrected for the population under 14 in that year, but to the extent that this correction differs in other years, my statements about year-to-year changes in consumption would have to be amended. That is quite right.

Caffeine taken by mouth is very rapidly absorbed. I have data on two types of experiment in which this was directly studied. Figure 28A is an unpublished experiment of our own, in which 300 mg of caffeine were given in decaffeinated coffee to ten young men. Blood caffeine was measured by the method of Axelrod and Reichenthal (3), which involves the complete extraction of caffeine from plasma at neutral pH into benzene, followed by re-extraction into very strong acid, and ultraviolet spectrophotometry. The extraction procedure is based on the fact that caffeine is uncharged in the region of neutrality. The method is fairly sensitive and quite specific in that it differentiates between caffeine and its metabolites, which is the most important differentiation one has to make.

You see that in these ten subjects the maximum caffeine level was reached within one hour, which is the earliest time we took samples.

Other experiments have shown that the maximum level is reached even sooner. The efficiency of absorption of caffeine can be estimated directly by comparing plasma levels after intravenous and oral administration, by direct analysis of the tissues of experimental animals, or by applying to the plasma levels a knowledge of the total aqueous volume in which the drug is distributed. All these methods have been applied, and they all agree.

Axelrod and Reichenthal have shown that in human subjects the plasma levels at one hour are identical, whether the drug has been given orally or intravenously. They also demonstrated in the dog (Table 5)

TABLE 5

Distribution of caffeine in dog.

84 mg/kg i.v. was given 3 hours before animal was killed.
Data of Axelrod and Reichenthal (3).

	Caffeine in tissue water mg/kg	Ratio tissue/plasma
Plasma	82.7	1.0
Red blood cells	83.0	1.0
Cerebrospinal fluid	78.2	0.95
Liver	91.7	1.11
Lung	71.5	0.87
Heart	77.0	0.93
Muscle	71.0	0.86
Kidney	82.1	0.99
Spleen	73.2	0.89
Brain	77.2	0.93

that after 3 hours equilibration, after an intravenous dose, all the tissues and the plasma have about the same caffeine concentration, and this concentration is very nearly the same, per kg., as the total administered dose. Thus caffeine equilibrates completely in the body water compartments of all tissues. There is certainly no reason to think the gonads would behave differently, although they were not examined in this study. Finally, analyzing Figure 28A, if we assume distribution into all body water of man, as in the dog, and take 75 kg. as the approximate weight of our subjects, 62 per cent of body weight as total body water, 5 mg/l the level after 300 mg ingested, we find more than three-fourths of the administered caffeine was absorbed, and there are some reasons to think this may be an underestimate. We

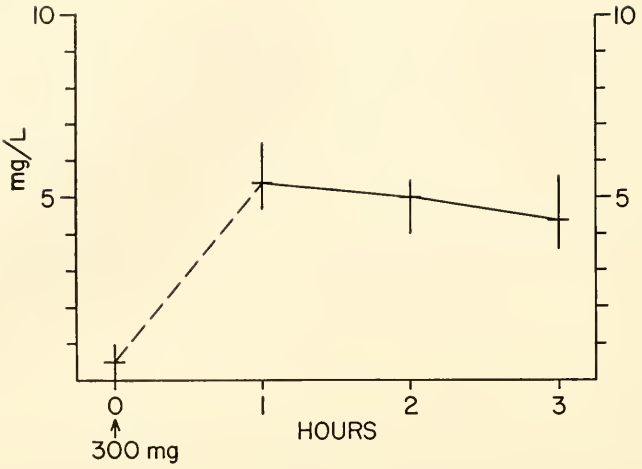


Figure 28A. Blood levels in man after caffeine administration. Caffeine added to decaffeinated coffee and taken by 10 subjects. Curve is drawn through mean values; range shown by vertical lines.

may summarize all this by saying that caffeine is very rapidly and essentially completely absorbed from the gastrointestinal tract, and is evenly distributed throughout the water phase of all tissues. This necessarily means that caffeine freely enters most (if not all) body cells.

Figure 28B depicts an experiment of Axelrod and Reichenthal, in which two cups of coffee at a time (caffeine content not measured) were repeatedly administered to human subjects, to find out about cumulation and persistence of caffeine in the body. You see that the subjects drank 8 cups of coffee between 8 A.M. and 6 P.M. As we would expect from the slow decline of the plasma level in Figure 28A, we see that repeated doses raise the plasma level higher and higher, because the rate of administration exceeds the rate of elimination. The level rises to about 4 mg/l and then falls slowly when no more caffeine is given. It can be calculated from the falling slopes in both experiments that the biological half life is about 3.5 hours. After a day of moderate coffee-drinking, the level would fall overnight to a very low point, and without further intake would fall practically to zero the next day.

It is known, chiefly from the studies of Cornish and Christman (16),

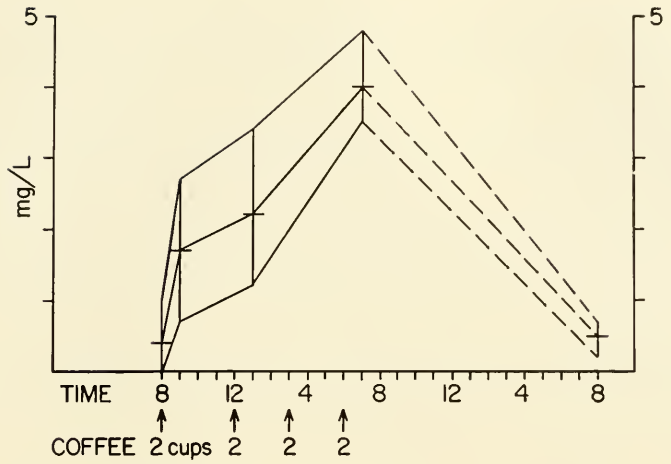


Figure 28B. Blood levels in man after caffeine administration. Coffee given repeatedly to 5 subjects as shown. Symbols have the same meaning as above.

that caffeine is almost completely metabolized. Practically none can be recovered in the urine, at least no more than 2 to 3 per cent of the amount ingested, but the major fraction of the ingested caffeine appears as metabolites. There are two kinds of metabolic alteration: removal of methyl groups, first from position 3, then from position 7; and also oxidation at position 8 (Fig. 27). The end result is a series of progressively demethylated xanthines, and another series of uric acid derivatives in which more or less demethylation has occurred. The principal metabolite of caffeine is 1-methyl uric acid, in which only the methyl group in position 1 remains, and position 8 has been oxidized. Minor metabolites include 1-methyl xanthine and 3-methyl uric acid, and traces of the other possible compounds. The pattern of metabolism of theobromine is somewhat different, principally in that a significant amount of theobromine itself is excreted. But I want to point out again that the caffeine levels we are looking at in these figures represent caffeine itself and not metabolites of caffeine.

Freese: How have these metabolites been measured? I mean, is it sure that these are the only ones?

Goldstein: I think it is quite sure, Ernst. These metabolites were measured by a good paper chromatography separation of products in the urine, and quite well identified quantitatively. This was done, I

think one should say, in only two human subjects, so it does not take into account the possibility of significant variations in the metabolic pattern from person to person.

As far as I am aware, we don't know anything about the mutagenic activity of the metabolites of caffeine, so we don't know how to estimate, really, whether falloff in the blood level is to be taken as a diminution of mutagenic activity, or whether, as the caffeine level falls and the metabolites come up, we may even be getting more mutagenic effect. This is something that should certainly be investigated. I will assume for the moment that only caffeine itself is mutagenic.

What we then, obviously, have to calculate is the mean levels to which individuals who are ingesting given amounts of coffee or tea are

TABLE 6

Coffee-drinking habits of 167 medical students at Stanford.

Cups per day	% of sample
0	23
1	17
2–4	37
5 or more	23

(Unpublished data of Goldstein and Warren.)

exposed. If you start with the population of the United States and the average consumption per capita, and correct for the number of individuals under age 14, you come out with 121 million people of coffee-drinking age, with an average consumption of about 23 pounds, or 10 kg. The average caffeine content of coffee is about 1 per cent. This would give us an exposure of about 100 grams of caffeine per year per capita or approximately 300 mg a day. That amounts to 2 to 3 cups of coffee in a day.

That this is a reasonable result we can judge from information about actual coffee-drinking habits. Table 6 presents a small sampling of such data from a study we did on Stanford medical students. This group may not be representative of the population as a whole,* but what we find is that about one-fifth of the sample doesn't drink any

*These data are borne out surprising well by a nationwide survey conducted by Elmo Roper in 1947-48 (51).

coffee or tea at all; another one-fifth drinks five or more, and sometimes up to ten cups a day. The bulk of the people drink one, two, or three cups a day.

If we accept 300 mg/day as the mean intake, we find this would establish 6 mg/l in body water, with a half-life of 3.5 hours. Integrating the exponential decay curve, we find a mean level of something like 1.3 mg/l, considered to apply over the whole 24-hour period. We may say, then, that the exposure of the population is as though all the human germ plasm of this country were constantly bathed in caffeine at a concentration of about 1 mg/l.

More realistically, we note that about one-fifth of the adult population is not exposed to caffeine at all. The majority drink between one and five cups daily, achieving a mean tissue level around 1 mg/l. Another one-fifth are heavy coffee-drinkers, who may be exposed to mean levels as high as 5 mg/l. These people are "addicted" to caffeine, in the sense that they clearly take caffeine for its psychic effects, they crave it strongly, and they display distinct withdrawal symptoms, such as headache (21), if it cannot be had.

We believe that coffee-drinking patterns are quite consistent for individuals; they remain stable over long periods of time, and we think they may in some respects be genetically determined. We have been interested for some time in the general belief that some people are kept awake by coffee and some people are not. When you talk to people about it, you get the impression that if they are correct about their own reactions to caffeine, there must be very great differences in the sensitivities to caffeine within the population.

We have been making studies, using large numbers of medical students as subjects, to try to get more information about these differences. The first question was whether it really is true that some people are consistent reactors and some people are consistent nonreactors to the psychic stimulation of coffee. We can now say with some confidence that this is certainly true. The criteria that we used were rather crude, because there is still no adequate objective test for psychic stimulation by a drug. But what we used—

Glass: You are probably not familiar with the little study, but I developed a test for the effect of caffeine on individuals, using a muscular and visual precision test, and applied it to two monozygotic twins, in a series of studies (27). They showed almost identical reactions when they were tested, and tested without knowing the other's reaction, in separate rooms, with placebos for the control and so on.

Benzer: You did find differences from one pair of twins to another pair of twins?

Glass: I had only one pair of twins to work with. I tried the same test on a group of students in a class and got all sorts of results, with no apparent consistency at all. But these subjects were not carefully diagnosed as to coffee-drinking and noncoffee-drinking individuals, so the interpretation is loose. In so far as the two monozygotic twins were concerned, they were not used to drinking coffee, and they reacted exactly alike over a period of about two to two-and-a-half hours, after ingesting eight grains as a dose.

Goldstein: Eight grains is approximately half a gram.

Glass: Yes.

Benzer: Is there any age dependence?

Goldstein: I don't have data on age dependence, because our subjects are all within the age of the early twenties. I would like to comment, though, in respect to what Dr. Glass said, that on the whole it has been very difficult, in the experience of psychologists and pharmacologists, to develop objective tests which will reveal the mild psychic stimulation obtained by people from drugs of the type of caffeine and amphetamine, except under conditions of prolonged sleep deprivation and fatigue. In that case there is ordinarily a decrement in the ability to perform various tasks, which can be partially restored by caffeine or amphetamine. A sleep deprivation procedure would not be useful for screening large numbers of people. We are working at developing a more practical test.

The criterion that we have used so far to distinguish reactors from nonreactors is a reliable one but clumsy. It is based on establishing standard conditions of caffeine deprivation from noon on a particular day until bedtime. Then a standard dose of caffeine is administered in decaffeinated coffee, in a double-blind, placebo-controlled design. We then ask the next morning whether the individual did or did not have difficulty falling asleep. This seems very crude, but with sufficient numbers of individuals and with a proper experimental design, you get quite statistically significant differences between caffeine and placebo. I might add that we were able to develop a method for adding caffeine to decaffeinated coffee so there was no perceptible difference in taste or appearance.

A reactor is classified as an individual who, for a long period of administrations, night after night, in a random design, in every case responds to caffeine by a disturbance in his ability to fall asleep, and in every case is not so affected by placebo. The nonreactors are those

who are never disturbed by either caffeine or placebo under these same conditions.

Our initial hypothesis was that the difference between reactors and nonreactors might well be in the absorption of caffeine from the gastrointestinal tract into the blood stream. This would not have been too surprising and would have been convenient, but it turned out to be entirely untrue. There was absolutely no difference in the rate of absorption or the blood levels achieved in these two groups. Presumably, the difference is referable to some properties of the brain itself or the penetration of caffeine to sites of action in the brain. Whatever the reason may be, there are important differences in the way people react to caffeine and in the extent of their desire for the effects of caffeine. These factors play a role in determining whether a person becomes a heavy coffee drinker. The reason why I stress this is that it makes it a little easier to consider what Dr. Neel rejected out of hand before, namely, the possibility that within a human population, one could define caffeine-exposed and caffeine-nonexposed individuals reliably enough to permit a comparative investigation of the incidence of genetic disease in their progeny.

As to the access of caffeine to the gonads, there is no direct evidence about this in man. As I said before, the presumptive evidence would be that caffeine has access to the gonads and germ cells, just as it has access to all other cells in the body. One must ask also about access to the fetus and its germ cells during the sensitive early period we postulated this morning. I know of only one experiment to determine whether caffeine passes in to the fetus from the mother, and that is an old one, done in 1934 in the dog, with very high caffeine levels and late in pregnancy.

In that experiment (22), there was complete passage of caffeine from maternal blood to fetal blood, and equal caffeine levels in the maternal and fetal livers. This is not too decisive, however, because there is a feeling among people who have studied such problems, and there are some experimental data to indicate, that the placenta passes substances more easily later in pregnancy than earlier (60). There have been studies, for example (24), on the passage of isotopic sodium from maternal to fetal circulation in the human, in cases where pregnancy had to be terminated for one reason or another. These show quite clearly that the passage rate increases with time during gestation. It therefore must be left open whether in the early period of gestation, when mutagenic substances might be especially hazardous, they may also be less able to cross the placenta into the fetus.

It occurred to us that these were matters that could be approached experimentally—the matter of access to the human gonads and to the human fetus. Unfortunately, I do not yet have more than one piece of incomplete data to present, but we are going to go on with these studies, as the opportunity presents itself.

With regard to the fetus, what is required is a therapeutic abortion being performed early in pregnancy, by a transabdominal incision so the fetus would not be macerated. Caffeine must be given before the operation to establish a high plasma level.

With regard to the gonads, we have two approaches. There are cases in which there is surgical indication for removal of both ovaries. In those cases, we propose to have one ovary removed first, to use as a tissue blank, then to make an intravenous infusion of caffeine before removing the second ovary. In the male, we have a similar possibility, when there is metastatic cancer of the prostate, and the surgical indication is for removal of both testes.

We carried out such an experiment in one patient, an 82-year-old man with prostatic cancer.* One would have liked to do the experiment in a patient in the reproductive age group, where there would be much less atrophy and fibrous replacement of the testicular tissue. With patience, we will eventually find such a patient. In the present instance, under spinal anesthesia, the control testis was removed and then an intravenous infusion of 500 mg of caffeine was given. This is a normal therapeutic dose if one were using caffeine as a respiratory stimulant. It is equivalent to about four cups of coffee. Higher doses could safely be given.

After a period of ten minutes for equilibration, the remaining testis was removed. The blood supply of the second testis was not disturbed during removal of the first. Our analyses showed that the plasma level established was about 19 mg/l. The equilibrium level at complete distribution in the body water should have been approximately 13 mg/l, from which we deduce that complete equilibration with the body water of all organs had not yet taken place in the ten-minute period. This is borne out by the fact that in the tissue water we found a lower concentration than in plasma. Slow equilibration is to be expected on the basis of the blood supply of the testis. Tissues with the most abundant blood supply obviously equilibrate first, other tissues more slowly. The testis, compared with many other body tissues, has a relatively poor blood supply.

* I am indebted to Drs. Roy Cohn and Joseph Hart for their co-operation.

Unfortunately, there was also some uncertainty about the exact amount of caffeine found in this experiment. The analyses, after correction for tissue blank, showed at least 1 mg/kg and possibly as much as 7 mg/kg in the testis. Technical changes should improve the precision of future assays. The point here is not that there is a particular concentration of caffeine in the testis, but that caffeine unquestionably enters the human testis. The caffeine there was identified by its characteristic ultraviolet absorption spectrum, i.e., by the difference in spectrum between the blank and experimental tissue.

Does anybody want to stop me at this time on any of these points? These are fairly factual matters.

Motulsky: What was the frequency of reactors versus nonreactors?

Goldstein: The clear, strong, and consistent reactors in our experimental group run about 20 per cent of the group.

Atwood: Did that group contain most of the heavy coffee drinkers?

Goldstein: No, there is not a good, clear relationship between reactivity and coffee-drinking habits; in fact, the heavy coffee drinkers tend to be less reactive to a given dose of caffeine, which you can interpret in one of two ways. It may mean that they have become tolerant to the effect of caffeine. But it may also mean that because they are less sensitive, they have to be heavy drinkers in order to get the same psychic stimulation that somebody else gets with a lesser amount.

McKusick: Is there a real bimodality here or does it shade off?

Goldstein: We can't say that, because of the insensitivity of our methods. Clear reactors, this 20 per cent group, are easy to pick out. We could also pick out those people who never, under any circumstances in a series of repeated administrations, gave a positive reaction to caffeine. That was another 20 per cent or so. That left the whole middle group who gave variable responses, sometimes to caffeine, sometimes to placebo, which may or may not have meant something. We were unable to say. We also don't know yet whether a person who fails to react to a 300 mg test dose would react to some larger dose.

Freese: I would like to ask whether the metabolism of caffeine in bacteria is known.

Novick: Yes. *E. coli* bacteria do not metabolize caffeine in any detectable way.

Freese: It could be, e.g., that in humans caffeine is metabolized so fast that it cannot act, while in bacteria, it stays around and therefore is mutagenic.

Novick: I feel that many things could be.

Freese: I'm just mentioning this because by understanding the metabolism first, one may be able to realize the difference or similarity between humans and bacteria.

Goldstein: It would be a great mistake if the statement stands in the record that caffeine is metabolized so fast that we don't have to worry about it. The fact is there is a very large and significant amount of caffeine present, on an average basis, continually, and this amounts to approximately 1 per cent of the amount that was shown in Dr Novick's experiments to be mutagenic.

The final problem I should like to attack is this: Is it possible to extrapolate from the known mutagenic effects of caffeine in bacteria and in Drosophila, to obtain any kind of quantitative estimate of the potential genetic hazard of this drug in man? I don't think these calculations will be worth very much because in order to make them at all one has first to make a number of assumptions, any one of which might be quite false. Nevertheless, with tongue in cheek, I am going to proceed.

To begin with, I assume the following: (1) Caffeine mutagenesis, directly or indirectly, is an effect upon DNA. (2) The average susceptibility of DNA to caffeine mutagenesis is about the same in all species. (3) All genes of a given organism are equally susceptible to caffeine mutagenesis, or, if not, considering the large number of genes in an organism, one can at least speak meaningfully of an average susceptibility. (4) Caffeine mutagenesis is a linear function of dose. (5) Caffeine equilibrates freely between plasma and gonadal tissue and passes freely into the fetus throughout the gestation period. (6a) Caffeine acts continuously upon DNA, regardless of stage in the replication cycle, so the total effect is linear with astronomical time, *or alternatively,* (6b) Caffeine acts only during DNA replication, so the total effect is proportional to biological time (the number of replication cycles).

Novick: I would like to challenge one. Are you going to discuss them?

Goldstein: Let's discuss them now. I am making the assumption that caffeine mutagenesis has something to do with DNA, whether it acts on DNA itself, DNA replication, or some other cell process concerned with replication.

Benzer: It is a highly unwarranted assumption.

Goldstein: It's not really a necessary assumption to what we're talking about.

Benzer: Then, let's just cross it out.

Goldstein: Then I make the assumption that the DNA's of all species are equal, or at least the genetic effects on all species are about the same. Without this assumption, I can do nothing, because the only data that we have to work with are on bacteria and Drosophila. If we're going to make any first approximations to man, we're going to have to do it by assuming that the susceptibility to the mutagenic effect is the same in man as in these other species. I don't believe this assumption for one moment, but as a first approximation—

Benzer: You can cross that one out, too. Nobody else believes it, either.

Neel: If you cross that one out, you would have to cross out quite a few extrapolations about radiation effects. Are you willing to cross them out?

Auerbach: No.

Novick: The genetic material is the same in both cases, specifying the exact nature of these events.

Neel: Would you accept that correction—the genetic material?

Goldstein: Yes. I was trying to say, if we are to make any calculation at all, we must forget for the moment possible differences in susceptibility to caffeine between bacteria and Drosophila and man; otherwise, we would be talking about—

Lederberg: The listing of assumptions in this fashion lets you inquire where the biases might come in. That could include the best judgment you can make of the final conclusion. If you have no information that suggests that this ought to be modified in one direction or another, with respect to the final conclusion, we will all agree this is a statement about which there is considerable doubt. But we are unable to say whether we are making an over- or an underestimate of the final result. I think, however, it is important to expose where the biases might be, by making assumptions of this kind.

Auerbach: The second assumption does seem doubtful; as far as we know at present, caffeine produces mutations in bacteria and not in Neurospora.

Lederberg: Are you prepared to suggest whether human DNA is more or less susceptible to caffeine than bacterial DNA?

Goldstein: To emphasize what Josh says, I have made assumptions which I know are false, as well as assumptions about which there are doubts, precisely for the purpose of putting down on paper what the assumptions have been that underlie any calculation one is going to make.

On the one hand, we have data on the mutagenic effect of caffeine in

lower organisms. We have no data in man. If we are going to try to make any extrapolations, it will have to be done on assumptions that we know are false. The alternative is to stop here until we get evidence in man. I think nobody would suggest doing that.

Freese: But you don't have to make these assumptions. If you merely make the assumption that the genetic material in different species is equal or similar, and if you make the further assumption that the enzymatic pool of different species is similar, that is sufficient.

Lederberg: I have suggested a solution; that is to say, to put the dilemma, to start with, in so far as these considerations bear on the estimation of caffeine mutagenesis—

Benzer: Assumptions that don't bear on the question should be cut out.

Lederberg: Perhaps so.

Benzer: Why perhaps? What is the point of an assumption?

Neel: May I suggest that we permit Dr. Goldstein to develop his argument and then attack him when he has built up his case? In my opinion, he has been unusually careful in setting forth his assumptions, and they are no more nor less than have been employed frequently in the past ten or twenty years in making certain extrapolations.

Benzer: The conclusions would be no more valid, by the same token.

Goldstein: The fourth assumption is one that I mentioned earlier—that caffeine mutagenesis is a linear function of dose. We have to postulate this, because all the evidence that we have from lower organisms is obtained at a concentration which is orders of magnitude higher than the concentrations of caffeine to which man is exposed.

Novick: I think the assumption is a good one, because our experiments showed that it is linear down to 10 mg/l and extrapolates through zero.

Goldstein: I think that there is at least some evidence on assumption 5. It is probably true that caffeine has free access to the gonads, at least in the adult gonads and possibly in the fetal, although our data on this are still incomplete.*

For assumption 6, there are two alternatives. One is that the effect of caffeine exposure is linear with time, regardless of replication cycles. My comment is simply that this probably is true of chemically reactive agents which have been demonstrated to be mutagenic *in vitro,* but it

*Addendum: We have now shown that caffeine equilibrates freely between blood and gonads in the human male and female, and also that the concentration of caffeine in the human fetus at 6 to 8 weeks gestation is the same as in maternal blood plasma. Goldstein, A., and R. Warren. Passage of caffeine into human gonadal and fetal tissue. *Biochem. Pharmacol,* in press (1962).

seems improbable for caffeine. It should be pointed out, as I think Dr. Novick did this morning, that there might be a relationship with time, but it was, nevertheless, essential that bacterial growth occur during the exposure to caffeine or else one would not get mutagenesis; likewise, in his chloramphenicol experiments, there was no mutagenesis.

Novick: It is a simple enough experiment, I would imagine, to add P^{32} to see if it is incorporated into the DNA of spermatogonia. If it is incorporated, they are exactly like our bacteria, and mutations would be induced at a constant rate per hour.

Atwood: Kubitschek (35) published to the effect that caffeine-induced mutation rate is proportional to growth rate, but I don't think you believe that.

Novick: He said "under one circumstance."

Atwood: When the growth rate is limited by the carbon source. I think that was glucose.

Goldstein: To what extent do you think that your results may be related to the growth limitation by tryptophan?

Novick: We tried it for a wide variety of growth factors, including limitation by lactate and ammonia. In all these cases the induced mutation rate was a constant per hour. With bacteria, one can define two states with respect to growth: a growing state and a nongrowing state. The extreme nongrowing state is the spore or the resting bacterium. Here one can induce mutations with things like irradiation and nitrous acid, and not with caffeine. In growing bacteria, there is some kind of limitation, whether it be nitrogen source, amino acid, energy source, etc. Growth can be limited to very low rates and I think that such bacteria are very much like many of the cells in the higher organisms.

Glass: Is that growth apart from division?

Novick: Growth in the sense I use it is always accompanied by division.

Glass: That's what I thought in bacteria, but we may be faced with a different type of growth in the germ line.

Novick: I think the important point in all of our experiments is that where DNA is being synthesized, where P^{32} is being incorporated into the DNA, the system is susceptible to mutagenesis.

Goldstein: I would point out that in the male the spermatogonia, stem line spermatogonia, are growing slowly. This is accepted. In that system, it doesn't matter whether you talk about absolute time or biological time. It amounts to the same thing in the sense that there is a mean generation time. The real question is whether the time scale

that you find in bacteria is the same time scale that we are to apply to man, or whether the time scale is in some way linked to the division time in bacteria. This is a very important aspect. If it is really per hour, as will be shown in the next calculation, one gets extremely high estimates for the effect of caffeine in the 30-year exposure in man.

Lederberg: Isn't it a question of the mutation rate in man, expressed per cell per hour, compared with the mutation rate in bacteria per cell per hour, for the range of mutations you are concerned about?

Novick: Not for the range, Josh, but for the mutation you're talking about.

Lederberg: If you have information to suggest that the radiation equivalence for caffeine differs widely from the different loci in bacteria, that would have to be taken into account.

Novick: Yes, we do.

Lederberg: We would then have to concern ourselves with not only the over-all mutational rate, but also with the mutation spectrum of human tissues compared to the bacterial situation. I think we're always more concerned about those that occur frequently. As long as any substantial fractions of mutation rates are at the high range, the problem hasn't changed one bit.

Benzer: You can at least calculate the mutation to T5 resistance in man.

Novick: Right, or T6. I think the thing here is that one will come out with an embarrassingly high figure for mutational equivalence. The important thing right now is that I don't want to see that as the basis for assuming the spermatogonia are not sensitive to mutagenesis during the adult period.

Goldstein: I still don't feel we have reached a clarification of this matter of time. If you propose that the effect of the mutagen is per hour, per cell, and you then propose to take that same figure per hour, per cell—forgetting for the moment whether the gene you chose is a good one or a bad one—are you going to transfer that to man and say that the estimate is likely to be the same per hour per bacterial cell as per spermatogonial cell, or are you going to translate it into a time scale that would make the hour in bacteria equivalent to ten days in man or something like that?

Novick: The absolute value has no meaning. What we compare is the ratio between the spontaneous mutation rate and the induced mutation rate. We are going to compare the mutation rate produced by caffeine with that produced by X-rays, but its actual value has no meaning.

Neel: Would it not be appropriate to let Dr. Goldstein go ahead

with his assumptions and the calculations which follow and then explore what happens if we alter his assumptions along lines suggested by some of the comments?

Auerbach: May I just ask Dr. Novick one question? Couldn't your data be explained if one assumes that, dependent on the length of generation time, you have a constant fraction of this during which synthesis takes place?

Novick: Yes, of course. I don't say that mutation is produced at any time.

Auerbach: So it could be expressed as a fraction of generation time.

Novick: It could very well be.

Auerbach: That might apply to human cells, too?

Novick: I think it would make no difference.

Goldstein: I don't think there is any question about spermatogonia division here. I think this is a red herring. We're arguing about something else.

Lederberg: Did somebody propose that spermatogonia would not multiply?

Goldstein: I don't know.

Novick: Yes, in assumption 6a.

Goldstein: I don't think that is implied.

Novick: It is explicit.

Goldstein: Where this does come in, however, is in relation to the female, where the evidence, as we have heard, is that germ cell division does not continue beyond the fetal period. One would expect that in the female, however, agents which acted with time regardless of the process of cell division would still be effective. However, let's go on.

What we do next is to take Novick's data on the effect of 150 mg/l of caffeine which produced, in addition to the spontaneous rate, 17×10^{-8} mutations per hour per cell in the chemostat, at a generation time in those experiments of about 5.5 hours. In the same experiments, 90 *r* radiation per hour had the same effect.

First, we reduce the concentration by a factor of just over 100 to get it down to the level to which the average exposure in man corresponds. On the assumption of a linear dose relationship, this would give us 1.5×10^{-9} mutations per hour per cell in man. Since the data refer to a single gene, and I want to find out the total probability of any mutation in man, I have to multiply this by an estimate of the total number of genes in man, which I have taken as 10^4. This gives $1.5 \times$

10^{-5} as the total probability of mutation per hour per sperm. Then, using assumption 6a, and multiplying by the number of hours in 30 years (2.6×10^5), we obtain approximately 4 mutations per sperm at the end of 30 years due to caffeine exposure. In terms of radiation equivalent, we take 90 *r* per hour reduced by the same factor of about 100 to obtain 0.8 *r* per hour, which, on a 30-year basis, comes to about 200,000 *r*. Therefore, as you predicted, you come out with very high estimates.

Obviously, we are not to accept these estimates. I believe the main trouble here was using time rather than cell divisions as a basis for extrapolating from bacteria to man. Another trouble may be that we have chosen this T5 gene which may be entirely inapplicable to the situation in man's genes.

Novick: In the same paper, there is the same T6 resistance, which gives a figure ten times less than that.

Atwood: So it's only 20,000 *r;* is that right?

Goldstein: Yes, 20,000 instead of 200,000.

Atwood: That's not much better.

Goldstein: Perhaps we can do the other type of calculation based on assumption 6b, and then compare the two results.

Novick: I'm sorry, but I would like to make the further point that this paper also described the effect of caffeine on bacteria growing anaerobically. From these results you would have concluded that the radiation equivalent of caffeine is negligible since X-rays are still very mutagenic under anaerobic conditions. Here it appears that it depends upon whether you view man as an aerobic coli or an anaerobic coli.

Lederberg: By what factor must you reduce the observed rate aerobically?

Novick: From 10 down to .5, a factor of 20, at least.

Goldstein: To calculate the effect of caffeine per cell division cycle rather than per hour, we proceed as follows. We start with the same rate per hour in bacteria but with a generation time of approximately 5.5 hours. Per cell division, this gives a caffeine-induced rate of about 10^{-6}. Reducing this by a factor of about 100, to get down to the mean concentration of 1 mg/l in man, we have approximately 10^{-8} induced mutations per gene per cell per division cycle.

This would be the value of p in the equation I presented this morning (p. 180). Using the parameters derived there, we can calculate a caffeine-induced mutant frequency of 0.15 per cent in sperm or ova at puberty, caused entirely by exposure during the fetal period.

In the male who drinks a few cups of coffee daily beginning at puberty, we can calculate an induced mutant frequency of nearly 3 per cent by age 30.

If it is assumed that the average effect of radiation is to cause approximately 10^{-7} mutations per gene per cell per r in man (56), it follows that the calculated effect of caffeine on the stem line of spermatogonia ($p = 10^{-8}$) would be equivalent at age 30 to a cumulative dose of about 50 r. This is in the range of what is considered to be the probable doubling dose in man. A man who drinks as much as 10 cups of coffee daily would receive the equivalent of about 150 r. Each cup of coffee would contribute about 0.003 r. During the first trimester of pregnancy the effect on the fetus would be roughly equivalent to about 3 r cumulative exposure for average (3-cup daily) coffee intake during this critical period.

I would say to Dr. Benzer that I hate to be put in this position. I am not trying to argue anything for these assumptions. I think they are so tenuous that they probably should not have been made at all. But, in effect, this is what I was asked to do. There is a limited amount of data, very little is known, so you either do something or not. Perhaps it is more valuable not to do something until there are more data to suggest what should be done. We will accept that as a criticism. But I have chosen to do it the other way, to see what comes out. I fully agree that these are very uncertain estimates.

One can try the same calculations with Drosophila data. Andrew (1) published on sex-linked lethals, in which he found that the effects of caffeine were roughly equivalent to about 150 r for the doses he used, which were quite high.

In his first experiment he gave caffeine at 2500 mg/l in the larval food. The spontaneous rate was 0.14 per cent. He found 0.7 per cent lethals in the progeny of males from one to 11 days old. In the second experiment he gave a single injection of caffeine (probably around 400 mg/kg) to adult males less than 24 hours old. Here he obtained 0.6 per cent lethals, but no effect beyond the seventh day (brood 7).

Analysis of these data is difficult because in the first experiment we cannot estimate the caffeine concentration to which the larval gonads were actually exposed, since we know nothing about the rates of metabolism and excretion of caffeine in this case. The maximum concentration possible would be that in the food; using this figure we are likely to obtain a gross underestimate of the mutagenic efficiency of caffeine. In the second experiment the exact dose administered is not

stated, but one can assume with some reason that the amount probably injected was distributed into body water. Assuming that this initial concentration was maintained for a whole day, we can again obtain an underestimate of the caffeine effect, which, however, should not be as much in error as that from the experiment with larvae.

The Drosophila results, unlike those obtained with the bacterial chemostat, are not mutation rates, but final mutant frequencies in sperm. To use our equation to calculate p, the probability of induced mutation per cell per division cycle, we must have information about m_1, m_2, etc., for Drosophila. From the number of germ cells at pupation and the size of the sperm clones (55) we can estimate that there are about 30 cell generations in the lineage of the sperm. Since the effect measured may presumably involve any of some 300 genic loci on the X chromosome, we must let $n = 300$ in our equation. Finally, we have to reduce the caffeine concentration to 1 mg/l. The experiment with larvae then gives $p = 5 \times 10^{-10}$ as compared with 10^{-8} for the T5 gene in *E. coli*. As we have pointed out, this figure is necessarily an underestimate.

For the single-injection experiment we assume a level of 700 mg/l in the body water of the fly. Here there was no exposure to caffeine during the larval period; we will estimate that, on the average during this experiment, there were 10 division cycles of stem cells and spermatocytes. Calculation then yields approximately $p = 5 \times 10^{-9}$.

I think it is interesting that, rough as these estimates are, the Drosophila data, based on a large number of different genes, lead to a mutagenicity of caffeine that is not too very far from what the bacterial data say. No such experiments with caffeine have been done in mice or any other mammalian organism. I should think that the only way to get some sound evidence to replace these speculations would be to initiate some good experiments in mice, to see whether the extrapolations presented here have any validity at all.*

*Addendum: Lyon *et al.* compared the specific locus mutation rates in male and female mice which had 1 g/l caffeine dissolved in their drinking water up to the age of ten weeks, parents having been similarly treated from the time of mating. The mutation rate per locus per gamete was 0.44×10^{-5} in males and 0.0 in females; these do not differ significantly from each other, nor from the known spontaneous rates. In this experiment 64,000 progeny were examined. However, radiation equivalent less than about 25 *r* would probably not have been detected. Cattanach has obtained essentially negative results in studying the possibility that caffeine at 2 g/l in drinking water causes translocations in mice. Lyon, M. F., R. J. S. Phillips, and A. G. Searle. A test for mutagenicity of caffeine in mice. *Z. Vererbungslehre,* in press (1962).

Auerbach: We are doing this now with mice. Dr. Cattanach is going to give our mice coffee to drink. We are working on too small a scale to do the kind of specific loci tests that the Russells are doing. We can only test for dominant lethals and translocations. The difficulty there is that if caffeine should act only on spermatogonia, Cattanach may not be able to detect its effect, because most dominant lethals are screened out in meiosis, and translocations occur infrequently in postmeiotic cells. On the other hand, the Drosophila experiments suggest that caffeine produces mutations in postmeiotic stages.

Novick: I would like to raise a genetic question among the human geneticists. Do we not already have some suggestion of what the upper limit is in the human, in some sort of terms? We can then set up an irradiation equivalent that man is exposed to, that is, coffee-drinking man. How big can the caffeine effect be to go unnoticed?

Lederberg: It depends on the age dependence.

Novick: How much is due to radiation?

Lederberg: Who knows what the radiation efficiency is?

Neel: The NAS committee suggested that the doubling dose in man was almost certainly between 10 and 150 *r*, with the true value very probably to be placed in the 30 to 80 *r* range.

Novick: So this, then, is a likely caffeine dose.

Glass: There is some more recent evidence from mouse work (50) that tends to support 30.

Lederberg: These are subject to exactly the same sort of criticisms that have been brought up here; I mean, they are all the most reasonable extrapolations you can make. We don't know the mutation rate in man. We don't know the efficiency of radiation.

Neel: What is your estimate for the doubling dose for specific loci in mice?

Russell: I'm surprised at talk of *the* doubling dose. It depends upon the locus. It depends upon whether you are talking about spermatogonia, spermatozoa, or oocytes. It differs for chronic and acute irradiation, and so on. There is no *one* doubling dose. Even an attempt to make a quantitative average of some of these factors is not of much value, because there are qualitative differences involved, too. There are qualitative differences between the mutations induced in spermatozoa, spermatogonia, and oocytes, and between at least some of these and those occurring spontaneously.

Neel: Very well, let us consider male mice receiving chronic radiation to their spermatogonia, which would correspond to chronic coffee-drinking. The next question is at what rate is the radiation

being given? You would, presumably, give us two different doubling doses, according to the rate—

Russell: I wouldn't give you a single one, to begin with. I think you would have to get one for each locus, and then you would be subject to the possibility of qualitative differences. It seems to me, until we know a good deal more, we may be talking about doubling the frequency of apples when we are measuring oranges.

Lederberg: Are you suggesting that we should ignore the problem of radiation mutagenesis in man?

Russell: Not at all.

Lederberg: I thought not.

Russell: I think we should talk in terms of absolute effect, the effect produced by radiation. At the present time, it is very hard to relate this quantitatively to the spontaneous rate.

Glass: Theoretically, Bill, I think your position is wrong. What you are really saying is that we have no basis for arriving at an average figure, but a population of mice or human beings which is sensitive to radiation must have some over-all doubling dose.

Atwood: Certainly, an acute irradiation can't have a doubling dose, because you would have to choose at what age you would want to take the spontaneous mutant frequency to be doubled, and, if it is different with ages, as we think it must be—

Russell: Let me give you another example. One of the estimates of doubling dose that has been used is based on irradiation of Drosophila spermatozoa. In the mouse, we find that irradiation of spermatozoa gives a high frequency of deficiencies which involve two closely linked loci in our test. We haven't found any spontaneous occurrences of this kind yet, so the doubling dose for this particular effect must be extremely low.

Here we have a type of mutation which, in spermatozoa, is easily induced, but which has not yet been observed to occur spontaneously. I think we need to know a good deal more before we can compare radiation-induced and spontaneous mutation in a meaningful way. It is highly misleading, I believe, to try to put our present inadequate knowledge into one single doubling dose estimate.

Neel: Does that answer your question, Dr. Novick?

Novick: Yes.

Lederberg: I think we all understand the problem.

Russell: With regard to tests of mutagens in mice, I should like to say that some experiments would not be tedious. If a chemical mutagen is likely to do whatever it is that causes dominant lethals, we have

a test that can be made without a great deal of effort. A test of the effect of a particular chemical on the dominant lethal rate could be completed in a few weeks.

Atwood: If that were true, and the estimate of 200,000 *r* or 20,000 *r* or something of that sort were correct, is it also true, then, that coffee drinkers would be sterile? They may be sterilized by dominant lethals.

Russell: Yes, if it worked this way.

Atwood: They would be sterile several times over.

Russell: In the Drosophila data that you have summarized, it looks as though the mutagenic effect reported from the treatment of adult males occurs before the seventh day matings. In other words, you are getting caffeine-induced mutations when there is no replication, and not getting them after the seventh day matings when the cells involved may have gone through replication.

Goldstein: No, this injection experiment was not chronic administration. There was a single injection of caffeine in the one-day-old male.

Russell: Yes, but I was referring not to the administration of the caffeine, but to the germ cell stages that were exposed to it. Matings made up to the seventh day would include cells that had been exposed in which stages?

Glass: Spermatozoa and spermatids; nothing earlier.

Russell: So all the effect produced, if what was observed was indeed a real effect, must have occurred in cell stages where no replication was going on. Then the effect ceases for later broods of offspring. The summary says "beyond the seventh day."

Goldstein: It was carried on to the eleventh day.

Russell: Eleven days may not be long enough to include spermatogonia.

Glass: One must carry the testing beyond the tenth or eleventh day to test cells that were spermatogonia at the time of treatment.

Russell: Matings made between seven and eleven days will, however, involve cells that were exposed as spermatocytes and possibly some exposed as spermatogonia. Thus, the reported mutagenic effect of caffeine occurred in cells in which there was no replication, and was not detected in cells in which there was replication!

Neel: I must say that I'm getting a great deal of wry amusement from all this. I am one who has refused for some time to be pushed into calculations about the genetic effects of radiation on man, and have been vigorously criticized by a good many of my colleagues for dragging my feet. Now, what's happened?

Lederberg: I would like to react to Avram with a statement of

criticism. We are permitted to believe, without indulging in utter fantasy, that much or all of the spontaneous mutation rate in man could be ascribed to caffeine; that this at least should generate some concern that would motivate doing much more extensive tests for mutagenic effect of caffeine in experimental situations closer to man, for example, mammals such as mice, and, indeed, if there is anything to this, even if a small fraction of residual effect can be extracted from the calculations that are described here, we should be at least as alarmed about caffeine as a mutagenic agent in man as we are about radiation.

There are many hundreds of thousands of people who are greatly exercised and involved in radiation mutagenesis, and there are possibly one, two, or three, if that many, people who are equally involved in mutagenesis by these agents.

I would like to make another point; that is, the argument runs in another direction. Another very important contribution that Novick and Szilard made in the same system was the discovery of antimutagens. They could take untreated cultures and reduce the spontaneous mutation rate in certain loci by growing these organisms in the presence of adenosine. If we are so concerned about mutagenesis in man that we are willing to place serious constraints, at considerable cost and political effort, on industrial development in order to save the penalty of some few per cent increase over the background rate, we are equally well committing a drastic sin of omission in neglecting to conduct vigorous research on the possible reduction of the spontaneous rate by the use of such agents, which could accomplish very much more.

Russell: I thoroughly agree. And since there is some evidence, from the Drosophila data cited here, that caffeine is mutagenic in spermatids and spermatozoa, where no replication is going on, it should be worthwhile to test for dominant lethals in mice, both in males and females. If an effect is found, then, first of all, it would confirm that caffeine can act when there is no replication going on, and, at the same time—

Lederberg: I think you would agree that this is a rather weak experiment, and even if it showed no results whatsoever, the issue should not be in any sense considered closed.

Russell: Oh, certainly, a negative result would not rule out the possibility of a mutagenic effect. But if the Drosophila results are valid, something may show up in a dominant lethal test on mammals.

Atwood: Isn't the radiation sensitive volume for dominant lethals the biggest of anything that you can measure?

Russell: Yes.

Atwood: So that that would be a very good test. It would include a lot of the genetic material.

Lederberg: What is the dose rate dependence on lethals?

Russell: Dose rate dependence? We have tested this and found no dose rate effect for dominant lethals in postspermatogonial stages.

Novick: Is it linear with total dose?

Russell: Yes, as far as we can tell.

Magni: There is another possibility of directly testing the effect of caffeine. As you know, there are a few people who have tried to estimate the true average mutation rate in man, by different ways, from demographic data. Going back to the system that Cavalli tried to use, it should be just a question of asking another question in a demographic questionnaire: "How many cups of coffee did your father drink?"

Lederberg: Does everybody here know the system that Dr. Magni is talking about?

Magni: I think I mentioned it previously. I don't know whether it would be worth while to recall it.

Neel: Please do.

Magni: Yes. Cavalli (13) is analyzing statistically a very simple fact. He is taking into consideration the sex ratio of the progeny of women and correlating it with the age of their father at the time of their birth, in order to see if recessive lethals accumulate in gametes with increasing age.

Atwood: Sex-linked?

Magni: Yes, sex-linked recessive lethals. This has been done so far on a sample of 60,000 married women. The regression of the ratio males/females born alive to the woman, on the age of the woman's father at the time of the woman's birth has a negative slope, as expected, though not significantly different from zero; the same ratio among the stillborn has a positive slope (which is significantly different from zero), as expected.

At the moment, data are being collected from birth registrations, and at the end of 1960 there should be some 800,000 births available. I don't know, however, if it will be possible to have questions on coffee consumption added to the Italian census.

Goldstein: It might be worth pointing out, apart from the general heterogeneity of the population with respect to caffeine exposure, there are certain special groups within the population who do not expose themselves to caffeine, for example, the Seventh Day Adventists or

the Mormons, who constitute a sizable group, and it might be possible to make comparative studies with other groups in the same communities, where other conditions would be roughly similar.

I would also say that the idea of asking how much coffee the grandfather drank is not as absurd as it seems, because of the finding that coffee-drinking habits are stable in individuals and perhaps also in families. A person who knew his grandfather at all knows if his grandfather was a heavy coffee drinker. He can't tell you whether he drank one cup or two cups a day, but he can certainly tell you if he was a constant coffee drinker. He also knows if coffee was never drunk in the family. So you can pick out the extremes. You could, on a simple questionnaire, pick out these groups.

Magni: Although the age investigated is that of the maternal grandfather of the progeny, it is the woman who is usually interviewed and not the progeny, so that it is the coffee-drinking habit of the woman's father which is to be investigated, which would seem an easy proposition.

Goldstein: I would like to ask one question which I think should be settled here, if it can be settled. Would it be going too far, on the basis of our present knowledge, to suggest—and this would be to the medical profession—that there may be a valid reason why caffeine should be avoided during the early part of pregnancy?

As a practical step it would be fairly simple to achieve. If there were cause for a general campaign against caffeine in the way there was a campaign against cigarettes, we know already this is unlikely to be welcomed with great enthusiasm, because people who drink coffee heavily need the caffeine, and you will have to supply them with an alternative stimulant of some kind which is not mutagenic. But to abstain from caffeine during a fixed period of a few months is something that is not difficult to do, if it were suggested on medical grounds.

Russell: I think it would be too early to make this recommendation now. There is not yet any evidence from mammals, and the only evidence in animals that you have cited (Andrew's Drosophila experiment) does not seem to agree with the assumption on which your recommendation is based, namely, that caffeine acts only during DNA replication.

Spuhler: Among people who visit clinics for sterility problems are there more than the usual proportion of coffee drinkers?

Atwood: I don't think anybody knows about that.

Lederberg: I don't see the value of that type of data, though. I

can't conceive of your collecting an adequate sample to get an appreciable result. Do you really think you could?

Spuhler: The sample could contain several thousand per year.

Lederberg: But suppose all the spontaneous mutation rates in man were due to caffeine; do you still think you would be able to pick it up on the basis of this type of questioning?

Atwood: Certainly; it is a matter of common experience that coffee drinkers are fertile. An individual who is sterilized from caffeine must take an awful lot more of it than most people do. You would be able to tell by asking him.

Magni: There was a positive correlation between the age of the woman's father (at the birth of the woman) and the frequency of women with zero children in Cavalli's data. A sample of the size used by Italian Central Statistical Institute, as mentioned before, might perhaps be adequate for testing the problem.

Neel: Dr. Goldstein has reproached me for rejecting out of hand the possibility of studies on human populations, and perhaps I was overhasty, but at the moment I can't visualize any situation involving two groups strictly comparable in all the ways that you would like to have them comparable—one group ingesting a large amount of coffee and the other not. Perhaps somebody else can think of such a situation.

Magni: Are you asking whether it would be possible to find two identical groups?

Neel: As nearly identical as is possible, except in this factor of coffee consumption.

Lederberg: I don't see that you could do better than the Cavalli system in finding what heterogeneity there is in the population. It may be stratified in other respects, but the particular beauty of it is that you have been comparing not just two populations, but you are comparing the age dependence of the sex ratio, and it would at least give you some assurance that the difference you are speaking of is a difference in mutation rate. If you found a difference, you would then face the problem of whether it was the caffeine that differentiated these populations or whether it was something else that was associated, in some indirect way, with caffeine consumption. But you would be on much stronger grounds in discussing this in relationship to mutation.

Neel: I suspect that coffee consumption has strong socio-economic implications.

Magni: This can be taken into account because the questionnaire

used in the sample census of employment has, or can have, data which permit it to obtain information on socio-economical conditions of the people belonging to the sample. It covers the economic situation, employment, and so on. It is a complete census questionnaire, and every question can be put in the calculating machine, so you can do anything you want.

Lederberg: Would I be permitted to raise another but related issue? It may cut across caffeine.

Neel: Have you finished now, Dr. Goldstein?

Goldstein: I have extracted everything there is, I think. I will add just one point. Many pharmacologists are concerned about the indiscriminate use of drugs in the population, with respect to many potential toxic actions of drugs. I think it worth while to point out that although we still know very little about this, we could take what we do know as a further indication against the indiscriminate use of drugs on the ground that some of them might be potential genetic hazards.

Lederberg: I wanted to bring up a related point. There is a good deal of interest in this in view of the etiology of mongolism, and I think it may be apropos to indicate that there are a number of alkaloids—I don't remember whether caffeine is included among them—that have effects on the spindle of dividing cells. Indeed, if one were to calculate how one should experimentally produce mongolism in man, it would be by the administration of colchicine or other similar spindle inhibitors, presumably quite possibly immediately after impregnation, at the time of reduction division. I don't know at what time this applies in man. Do you know, Dr. Russell, just when reduction division takes place in the human egg?

Russell: In the mouse and, I believe, in man, sperm entry occurs when the egg is in metaphase of the second meiotic division. Thus, treatment after impregnation would presumably be too late to cause meiotic nondisjunction. However, important chromosomal disturbances can be induced during cleavage. Mrs. Russell (52) has been able to induce XO mice by irradiation between sperm entry and the first cleavage. She is also getting some evidence, although it is too early to say for sure, that XO can be induced by irradiation of sperm prior to entry.

Lederberg: Since there is the age dependence with respect to the mother in mongolism in man, an adult woman is presumably a target for such agents. Colchicine is used therapeutically; in fact, not so very rarely it is a specific for gout. It is one of the earliest specific

remedies that we have in the pharmacopoeia. As far as I know, no thought has been given to what bearing this might have, and to its genetic effects.

Besides colchicine, there are many other agents that are C-mitotic poisons in plants and cells. Colchicine is merely the most dramatic and the one most widely used experimentally. Chloral hydrate is another one which is a spectacular C-mitotic poison. I don't know whether it has been studied in animals, but certainly it has been used very extensively in plant material; in fact, even before colchicine came into vogue, it was used as a method of producing doubling of chromosomes. Ether has a C-mitotic effect, as does chloroform, and almost the entire range of what we call the nonspecific narcotics will do very much the same thing in animal cells. Many of these have been studied in nondisjunctional accidents. You get polyastral forms and so forth, by temporary suppression of the spindle.

I think there may be a legitimate cause for concern that such agents can indeed be responsible for nondisjunctional accidents in the egg, where, of course, they could be most consequential. One has to be concerned about using poisons of this kind generally since none of us wants very many of our somatic cells to be unbalanced in their chromosome makeup. At the time of the inception of the zygote, I would assume we would be acutely concerned because we now know what the consequences of chromosome nondisjunction would be.

Atwood: The maternal age dependence of mongolism shows that practically all the nondisjunctions are in the female, and I think that Dr. Stern has evidence that this is also true for Klinefelter's nondisjunctions.

Stern: The evidence for meiotic nondisjunction is less binding than we used to think. The age effect could possibly depend on mitotic nondisjunction in an early cleavage division. If so, one would have to assume that the eggs from relatively older ovaries have a tendency to undergo mitotic nondisjunction, perhaps of either maternal or paternal chromosomes.

Atwood: I mean, it was not an aneuploid sperm that did it?

Stern: In case of dependence on the age of the mother, chromosomal aberrations in the offspring are, of course, not caused by aneuploid sperm. But we cannot be sure either that it was an aneuploid egg nucleus which was responsible for the aneuploid offspring. There is considerable evidence in man for chromosomally mosaic individuals which must have arisen from mitotic nondisjunction. Even nonmosaic individuals with unusual chromosome constitutions may have

arisen from nondisjunction in early cleavage. The complementary chromosome constitution may have led to death of the blastomere, or it may have formed extra-embryonic tissue or adult tissue not accessible to chromosome analysis.

Atwood: Have the Klinefelter and Turner cases been investigated as to maternal age dependence?

Stern: Klinefelter's have, and there is a maternal age dependence which is not as strong as in mongolism.

McKusick: In Turner's, there appears to be no maternal age effect (11).

Atwood: It ought to be, because it is simply the complementary type to Klinefelter's.

Glass: No, this is not necessarily so, since you have the possibility of nondisjunction both in the male and the female. The frequencies of Turner's and Klinefelter's syndromes are sufficiently different that different mechanisms of nondisjunction might be involved.

Atwood: They are differentially hard to detect, though. Some of Klinefelter's individuals appear normal and might not be detected. Isn't that true?

McKusick: It is in the opposite direction. Klinefelter's are hard to detect and they are frequent. Klinefelter's syndrome is as frequent as 1 in each 400 male births (23). The Turner syndrome probably occurs about once in every 5000 births.

Stern: We do not yet know whether "chromosomally Klinefelter" individuals occur who are phenotypically normal, fertile males, and whether there exist also "chromosomally Turner" individuals who are phenotypically fertile females. After all, XO mice are normal and fertile. Unless we "survey 100,000 or so run-of-the-mill (human) females" (G. L. Walls, 1959, Peculiar color blindness in peculiar people, A.M.A. Arch. Opthal. 62:13-32) we will not have a clear idea as to the possible variation in the expression of the XO constitution. And the corresponding considerations would apply to the XXY constitution.

Neel: Is there further discussion? If not, I will turn the meeting over to our summarizer.

Stern: May I add one comment on the question of relative frequencies of Klinefelter and Turner types? Assume a zygote starts out with a normal XY constitution. Let there be mitotic nondisjunction of the replicated X chromosomes at the first cleavage division. Then, two cells will be formed, with XXY and OY constitution, respectively. The latter will die; the former develop into a Klinefelter type. Thus, this

mechanism will account for the origin of Klinefelter's without the occurrence of complementary Turner's.

Summary of Discussion

Zamenhof: The conclusion of today's meeting, as well as the other two days, would perhaps be best summarized in a story told us by Julian Huxley. At one meeting Huxley was approached by a gentleman of the press who asked him, "What are you scientists trying to find out?" His answer was, "All we are trying to find out is *something.*" Nevertheless, we have to publish details of today's meeting because, as Dr. Glass has pointed out, the thinking of other people may be influenced by the results of such a conference as this, a conference on an entirely new subject.

In the past, there was the misconception, as Dr. Goldstein has pointed out, that although the cell may be exposed to radiation, the homeostasis or the constant environment in the cell precludes the possibility of action of any chemicals. Perhaps because of this, although the public has been shown the dangers of increasing our mutational load by radiation, the danger of chemical mutagens as yet awaits its Linus Pauling.

Dr. Goldstein started his presentation by pointing out that many of the mutagens which were discussed during the past two days are actually used in everyday life, as drugs or otherwise, and therefore are worth discussing. Of course, all we can accomplish here is intelligent guessing, which is always subject to skepticism, but this is the best we can do at the present time, and we should do it.

Discussing in detail the substances which were implicated as mutagens, he mentioned that one of the drugs, phenoxybenzamine, is really a nitrogen mustard, and there is little doubt that nitrogen mustards are mutagens. Other potential mutagens are aldehydes, which are used as drugs; phenols; nitrites, used as preservatives in meats, and also as drugs and considered safe because people just don't care to be up-to-date with basic research; and atropine and morphine as well.

Dr. Glass pointed out that β-propiolactone is used as a drug to kill viruses; yet, as he has shown, this agent breaks chromosomes and has been shown to be a bacterial mutagen. Nicotine also has been shown to break plant chromosomes, although, as Dr. Auerbach has pointed out, plant chromosome breakage may not be the best phenomenon to test mutagenicity.

Dr. Goldstein was then concerned about the extent of exposure, and

he pointed out that the extent of exposure and time of exposure are very heterogeneous in our population. But his main thesis was that we can already prove that substantial contribution to spontaneous mutation rate can be pinned down to certain chemical mutagens and, in particular, caffeine.

Goldstein: I didn't say that we could assert that. I said that this was a possibility.

Zamenhof: All right, it is a possibility. Later, he tried to develop a formula for calculating to what extent spontaneous mutation rate could be accounted for by chemical mutagens; there were several assumptions necessary in developing such a formula and therefore this formula was subject to criticism. Nevertheless, it does not appear doubtful that the greatest danger would be during sensitive periods in pregnancy; in females after birth there seemed to be less danger, whereas in males, the danger is before birth as well as after birth, and of course, we don't know which of the two periods is more important.

Dr. Goldstein then classified drugs as to the extent of exposure, and divided them, rightly, into three categories: drugs to which people are exposed only occasionally and briefly; those to which special groups of people are exposed for long periods of time but other people not at all; and those to which practically all the population is exposed, for a long time.

Then a most interesting discussion developed, which I would like to quote here because it clarifies much of our thinking. Dr. Goldstein invited Dr. Novick to devise a standard test for estimating the danger of chemical mutagens, but Dr. Novick has declined the invitation because there could be no such standard test; further discussion showed why there could not be one.

Dr. Atwood suggested that the least we could do was to test whether a given chemical was radiomimetic, and Dr. Glass suggested that we use tissue culture for this purpose, as he already has done for β-propiolactone. Dr. Neel pointed out that we all agree that human tissue would be the best, but human tissue is the most difficult to study. The problem here, as presented to Dr. Novick, was whether we could find any other system, such as a bacterial system, which could serve as a good test system, though one realizes that there are difficulties in extrapolating from bacteria to man.

Dr. Lederberg suggested that, just for a list of suspected chemical mutagens, the use of Novick and Szilard's chemostat seems to be most appropriate, but no more than for a list of suspects. Human tissue should be used and should be of more value, but there is another

difficulty inherent in such a system: human cells are diploid and therefore one cannot detect recessive mutations. He suggested that either a search be made for haploid tissue, if any such thing exists, or some special tricks, such as using only males and sex-linked mutations.

Dr. Goldstein pointed out that it would be of practical value, even right now, if we had several drugs which have about the same effect. We should avoid those drugs in this list which have been shown to be mutagens because, if we can avoid them without sacrificing the therapeutic value, why not do it?

He then mentioned that what worries him in particular is that although caffeine may be a weak mutagen, perhaps there are some other potential dangerous drugs or chemicals which are still undiscovered, and perhaps we should search for those. He has given a list of such chemicals which are used by large portions of the population for a long time; the list includes ethyl alcohol, which *a priori* looks innocent but is not so innocent when we realize that it is changed in the normal metabolic process into acetaldehyde, which is not very unlike the mutagen formaldehyde. Nicotine has been shown to produce chromosome abnormalities in plants. There is a large group of chemicals which are in constant use, such as additives to water supplies and foods, insecticides, etc. Their mutagenicity has not been proved, but as he mentioned before, there too, some mutagenic agent could be lurking in the dark.

He also mentioned vitamins, and there I reported in a few words the mutagenicity of ascorbic acid. Although ascorbic acid by itself may not be very interesting as a mutagen, the fact that some, if not all cells, cannot live without ascorbic acid makes it important to study this vitamin and the whole class of natural mutagens which are present in normal cells. I gave an example of Novick and Szilard's pioneer research in which they found adenine to be mutagenic.

Going into details on caffeine, Dr. Goldstein first mentioned that this purine does not act by being incorporated in DNA, because that has never been reported. He has quoted statistics of the use of caffeine, exposures of gonads to caffeine, and he actually made measurements of distribution and proved that caffeine is accumulated there. Although the caffeine is eventually metabolized, the biological half life being around 3.5 hours, this period is long enough for caffeine to exert an effect; in addition, we cannot even prove that the products of normal metabolism of caffeine are harmless.

There was a question as to whether caffeine, which can actually reach the gonads—and he quoted experiments which seem to prove that it did—can pass through maternal blood to fetal blood.

In the case of penetration to testes, the accurate measurements may be due for correction, one way or the other. But this is not important. The important thing is that caffeine actually reaches this tissue.

Then he proposed to calculate just how much effect caffeine could have, especially as compared with radiation, and he had to make certain assumptions. These assumptions were subjected to the criticism of many members of the audience until Dr. Lederberg and Dr. Neel pointed out that unless we make some assumption, we cannot start a discussion of the problem; it was felt that perhaps it is better to bring things into the open, showing all the difficulties involved, because someone may remove the difficulties later on.

Dr. Goldstein calculated that the mutagenic effect of caffeine in 30 years could be as large as that of a radiation dose of 200,000 *r*. On the basis of this calculation he suggested that even though the figures may be due for very drastic change, it could be recommended, at least as a precaution, that pregnant women take no caffeine. The audience, or at least Dr. Russell, felt that perhaps it is still too early for this recommendation.

Goldstein: May I make a correction? The calculation that led to the figure of 200,000 *r*, or 20,000 *r*, whatever you want it to be, is not a calculation in which I place any confidence at all; in fact, I presented it only to show that if you take a per-hour basis and translate it to man, you get what seems to me an absurd figure. The figures that I think are possibly closer to the truth are the figures that are derived on an entirely different basis, which led to figures of the order of 50 *r* for a 30-year exposure, or something like that.

Zamenhof: There were some other suggestions made, which are less drastic. If caffeine is really so mutagenic, perhaps it would also produce sterility; sterility clinic could furnish some statistical data on this subject. Dr. Lederberg has brought up a point that many of the alkaloids may be dangerous, not because they are mutagenic but because they are spindle inhibitors, and would form chromosome nondisjunction or mongoloids; caffeine might have been on the list of substances potentially dangerous in this respect. An adult woman would be a good target for such action. Colchicine or other agents were also worth investigating. Dr. Lederberg then made the suggestion that we should be alarmed by Dr. Goldstein's presentation, and the least we could do is to continue research on this subject.

Dr. Lederberg also pointed out that there should be more research, not only on the subject of mutagenesis, but also on antimutagenesis; as we all know, Dr. Novick has discovered that there is such a thing (28,

45), and the phenomenon may be just as important, especially to counteract the effect of radiation.

Novick: That should read "Novick and Szilard," for the record, instead of the present statement of only Novick.

Zamenhof: If I may add one word more, I would rather subscribe to what Dr. Goldstein said, that there may be other dangerous agents lurking in the dark. One example is acetaldehyde, which nobody would suspect until it was pointed out. Perhaps caffeine is not the most important. Perhaps this calculation is not the most important. But it is important to have a case where these things have been brought to the scientist's attention.

Goodgal: Scientists have the responsibility not to raise issues that are not based on fact. If it is demonstrated that caffeine is a mutagenic agent in a large variety of organisms, and under a variety of conditions, or one can at least define them, then I think there is a much more solid basis for raising this issue. My own feeling is that there has been too much said today on too few facts.

Lederberg: This is not a completely new issue. It has been brought up before. I wrote a letter about it to the *Bulletin of Atomic Scientists**

* Text inserted at Dr. Lederberg's request in lieu of informal discussion at the conference:

To the Editor:

Several writers, notably Professor Curt Stern, have emphasized that any use of atomic energy entails a calculable risk, no less than those features of modern technology that lead to auto accidents and gastric ulcers. Nuclear warfare poses such an immediate and overwhelming peril to simple survival that concern for the ultimate genetic hazards of atomic energy betokens an almost unwarrantable optimism for the maintenance of world peace, but an optimism that is our only constructive recourse. However, if we postulate survival, we cannot overlook the long run genetic problems entirely for preoccupation with the narrower issues of public affairs.

As the bulletin shows, the attention of the informed public is rightly focussed on the production of deleterious mutations by penetrating radiations, but this emphasis may have obscured the possibly wider contact of genetic hygiene with industrial civilization. Radiobiological discussions have often taken the spontaneous mutation rate as a reference base, as an unavoidable evil which could not be averted and ought not be aggravated. However, recent studies have established two relevant facts: 1. A variety of chemical reagents can also induce mutations. Many of these compounds are special drugs, but the list also includes such common substances and natural metabolites as formaldehyde, hydrogen peroxide, and caffeine. 2. Still other chemicals can reverse these mutagenic effects and can also reduce the "spontaneous" mutation rate. Much (but by no means all) of this research has been conducted with microorganisms and more extensive studies are needed to establish, for example, whether the germ cells of man are physio-

five years ago, and there has been other discussion. The fact remains that, without generating a minimum of public excitement or at least excitement among the scientific community, nothing is going to be done about it. It is too easy to think that the results we get in the laboratory concerning mutagenesis in bacteria (33) are merely scientific curiosities and that they can be excluded from the realm of human affairs. This is too convenient.

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logically insulated against such chemical insults from the environment. On the other hand, it may be possible to ameliorate the intracellular biochemical accidents that can now plausibly be considered as one source of "spontaneous" mutations.

From this perspective, the genetic hazards of atomic energy are but one facet of a much broader and correspondingly more urgent problem of chronic toxicity and the health of the public (and its future generations).

Joshua Lederberg
October 12, 1955

P. S. In the above discussion, survival is "postulated." This is, of course, far too passive a response to such an urgent threat. The postulation is intended not to encourage passivity, but to focus on the immediate issue. It should also be clearly understood that the broadening of the basis of genetic hazards does not in any way mitigate specific dangers from atomic energy. The role of radiations, and public response to it, may perhaps be compared to the role of poliomyelitis as one of many contagious diseases that are important in public health.

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INDEX

ABO, 16-46
Acatalasemia, 124
Acetaldehyde, 172, 195
Achondroplasia (Chondrodystrophy), 14-15
Acriflavine, 80
Adenine, 93, 95, 97, 102, 155, 185, 202
  deamination of, 82
  requiring strain of Neurospora, 131, 134
Adenine reversions, 133
Adenosine, 226
Adenosine riboside, action of formaldehyde, 114
Adrenergic blockade, 171
Agammaglobulinemia, 23
Agents, alkylating, 168, 171, 175, 177
  carcinogenic, 170
  carcinostatic, 170
  mutagenic, 170
Agglutinogen sites, number of per cell, 33
Aldehydes, 171
Alkylating agents, in Hodgkin's disease, 170-71
Allyl *iso*thiocyanate, 79, 197
Amino acid peroxides, 114
Aminopurine, 95-96, 149, 152
  incorporation into DNA, 89
  incorporation of, in phage T4, 91
  mutagenic action of, 96
  mutants, induced, 104
Aminotriazol, 197
Amphetamine, psychic stimulation of, 210
Anti-A_1, Dolichos, 28, 39
Antibody, bi-specific, 33
  fluorescent, 26-27
Antimutagenic, effect of purine riboside, 98
Antimutagenic nucleoside, 177
Antimutagens, 115, 177, 226
Antirrhinum, mutation of two alleles in, 57
Ankylosing spondylitis, 175
Anoxia, 174
Ascorbic acid, 198, 201
Atropine, 169, 173
Autopolyploidy, cryptic, 69
Azaserine, 89, 98

B vitamins, 198
Bacillus subtilis, double mutants in, 54
  tryptophan and histidine markers in, 55
Bacteria, drug resistance in, 190
  effect of caffeine on, 220
  heminless, 130
  hot spot in, 144
  induction of mutation in, by nitrous acid, 86
  induction of mutation in, with heat, 86-87, 149
  metabolism of caffeine in, 213
  mutagenic effects of caffeine in, 214
  mutagenicity of heavy water in, 98-99
  mutations, expression in, 70
  recessive losses of function in, 5
  treatment of, with nitrous acid, 85

Benzpyrene, 196
Beta-propiolactone, 110, 173, 187, 233
Blood groups, ABO, 16-46
Bombay factor, 20, 22
Bone marrow, number of stem cells in, 41
Brain, conservation of DNA in, 184-85
Bromodeoxycytidine, 149
Bromouracil, 65, 88, 92-93, 149, 151-52
as a deoxynucleotide triphosphate, 94
incorporation into DNA, 89
mutagenic action of, 92

C-mitotic poisons, 231
Caffeine, 89-90, 98, 176, 179, 188-89, 194, 200-201, 203, 219
absorption of, 204-6, 211
access of, to the gonads, 211-12, 216
and spontaneous mutation rate in man, 226
concentration of, in the human fetus, 216
consumption of, 208-9
cumulation and persistence of, in the body, 206
DNA replication and, 228
effect of, on bacteria, 220
effect of, in Drosophila, 221-22, 225
exposure to, 203
induced mutation rate, 217
metabolism of, 207
metabolism of, in bacteria, 213
mutagenic activity of the metabolites of, 208
mutagenic effects of, in bacteria, 214
mutagenic effects of, in Drosophila, 214
mutagenicity of, in mice, 222-23
penetration of, to sites of action, 211
psychic stimulation of, 210
radiation equivalence for, 218, 220-21
reaction to, 210-11, 213
sensitivities to, 209
testing the effect of, 227
Caffeine mutagenesis, 214
linear function of dose, 216
Carbon monoxide, 198
Carcinogens, 196
Catalase, 123-25
Catechols, 172
Cell, reactive sites on, 34
Chemical mutagens, a standard test for, 234
Chlamydomonas, amino acid requirements in eugametos, 30
amino acid requirements in Reinhardi, 69
Chloral hydrate, 169, 172, 231
Chloramphenicol, 217
effect on protein and nucleic acid synthesis, 60
Chlorine, 196
Chlorogenic acid, 201
Chlorouracil, 92
Chondrodystrophy (Achondroplasia), 14-15
Christmas disease, 71
Chromosomal aberrations, dependence on the age, 231
Chromosome aberrations, 35, 65, 174-75, 187-88
Co-agglutination, 26, 34
Coal-tar dyes, 197
Coffee-drinking, patterns of, 209
reactions of twins to, 210
Colchicine, 230
Cross-linkage, 79
CysC-mutants, deletions among, 118-19
Cytosine, 89, 155
deamination in viral RNA, 59

DDT, 197
Deamination, of adenine, 82
"Deletions," 104, 118
Delphinium ajacis, unstable genes in, 51
Deoxyguanosine, 113
Deoxynucleotide triphosphate, 94
Deoxyribonuclease, 81
Diaminopurine (2, 6-Diamino), 97, 149, 151
Dictyate stage, 183
Diepoxides, 187
Diepoxybutane, 80, 105, 109, 131-34
lethals and translocations in Drosophila, 110

Diethyl sulfate, 100, 102-3, 113
alkylation of guanine by, 177
Digitoxigenin, 169
Dimethyl sulfate, 101, 113
DNA, conservation of, in brain, 184-85
conservation of, in liver, 184-85
incorporation into aminopurine, 89
incorporation into bromouracil, 89
inosine in, 98
replication and caffeine, 228
DNA polymerase, 90, 144
Doubling dose, in man, 223
in mice, 223-24
meaningfulness of, 223-24
Drosophila, diepoxybutane, lethals and translocations in, 110
effects of caffeine in, 221-22, 225
heterochromatic segments in, 122
mutagen specificity in, 128
mutagenic effects of caffeine in, 214
mutagenicity of heavy water in, 98
somatic crossing over in, 56
Drosophila funebris, mutants, visible in, 69
Drosophila virilis, unstable genes in, 51-52
Drug resistance, in bacteria, 190
Drugs, genetic hazards of, 168

EDTA, 116
Enovid, 199
Epinephrine, 169, 172
Escherichia coli, amino acid starvation in auxotrophs, 62
galactose region of, 4
lysine reversions, 135
manganese chloride, mutagenic effect in, 115-17
mutator strain of, 68
phosphatase system in, 159
reversions at the histidine locus in, 134
single and double mutations in, 53, 55
tryptophan reversions in, 133
tryptophanless strain, 131
UV induced mutations in, 132
Escherichia coli B, T4-resistance in, 202
Estradiol, 200
Ethoxycaffeine, 98
Ethyl alcohol, 195
Ethylated guanine 108-9, 113
Ethylene imine, 67, 79-81, 110
Ethylene oxide, 80, 109, 173
lethals and translocations in Drosophila, 110
Ethylethane sulfonate, 100, 102, 105-8, 111-12, 131
action on phage, 111
Ethyl guanine, 101
Ethylmethane sulfonate, 100, 103, 107, 149, 151
Ethyl sulfate, 67

Ferrous chloride, 116
Ferrous sulfate, 116
Fertility, relative, 5
Fluoride, 197
Formaldehyde, 80, 114, 134, 172
action of adenosine riboside, 114
Furfuryl alcohol, 201

Garlic extracts, 197
Genes, penetrance of, 34
unstable, 3
"Gene conversions," 135
Ghosts, red cell, 23
Gland, shell-forming, 46-47
Glucosylation, phage T4, within the *rII* region, 143
Glyceraldehyde, 202
Gonads, access of caffeine to, 211-12, 216
Grigg effect, 135
Guanine, 89, 93, 103, 155
alkylation of, 105
alkylation of, by diethyl sulfate, 177

Heat, induction of mutation in bacteria with, 86-87, 149
Heavy water, mutagenicity of, in bacteria, 98-99
mutagenicity of, in Drosophila, 98
Heliotrin, 79
Hemophilia, 6
Hepatitis virus, 174
Heterochromatic segments, in Drosophila, 122

Histidine, 54
locus, reversions at, in *E. coli*, 134
Histidine markers, *Bacillus subtilis* in, 55
Histidine system, mutations in, 3
"Hit poison," 99
Hodgkin's disease, alkylating agents in, 170-71
"Hot spot," 136, 156, 160
in bacteria, 144
and specific mutagens, 150
spontaneous, deletion, 148
Hydrocortisone, 169
Hydrogen peroxide, 114, 116, 124-25
Hydroxylamine, 83, 143, 149
Hydroxylmethyl cytosine, 96, 142-43
deamination of, 82
Hypoxanthine, 97, 201

Indouracil, 92
Induced mutation, probability of per cell, 222
Induced mutation rates, in different gametogenic stages, 127
Inosine, in DNA, 98
Inositol, requiring strain of Neurospora, 131, 134
Inositol reversions, 133
Insecticides, 197
Intragenic suppressors, 160
Iron salts, 198
Isopropyl methane sulfonate, 67

Jellies, contraceptive, 199

Klinefelter's syndrome, 232
maternal age dependence of, 232

Leaf tissue, growth patterns of, 51
Lectin, bi-specific or heteroligating, 32
"Lethal hit," 112
Lethal mutations, in Neurospora, 60
Lethals, sex-linked recessive in man, 227
Leucine, mutants, 4
Leucine reversion, 133
Leukemia, 34
Liver, conservation of DNA in, 184-85
Lysine, locus, reversions at, in *E. coli*, 135

Maize, sectorials in, 51
variegation in, 49
Manganese chloride, 115
mutagenic effect in *E. coli*, 115-17
mutagenic effect in penicillin, 117
Manganese salts, 198
Manganous acetate, 116
Manganous sulphate, 116
Methemoglobinemia, 173
Methenamine, 169, 171
Methionine, 130-31
cistron in yeast, 53
Methylated guanine, 113
Methyl guanine, 108
Methylmethane sulfonate, 112
Methyl uric acid, 207
Methyl xanthine, 207
Mice, doubling dose in, 223-24
mutagenicity of caffeine in, 222-23
reduction division in, 230
sex-linked lethals, 191
tests of mutagens in, 224
XO, induced by irradiation, 230
Microorganisms, mutagen specificity in, 129
mutation rates in, 55
peroxidase in, 123
Mitosis, phytohemagglutinin-induced, 186
Mongolism, 15, 65, 230
maternal age dependence of, 231
Morphine, 79, 169, 172, 186
"Mosaics," 39, 40-42, 86
Mosaics, $A_2 + 0$, 36
Mussels (*Mytilus edulis*), 46
blue pigment, 46
cell lineage in, 48
free-swimming larval stage, 47
mutation rate in, 46-51
Mustard gas, 79
Mustard seed, 197
Mutagenic action, of bromouracil, 92
Mutagenic activity, 175
meaningful tests for, 168
metabolites of caffeine of, 208
Mutagens, reactive to pretreatment, 116
tests of, in mice, 224
also see specific chemical
Mutagen specificity, 126, 132, 135-36
in Drosophila, 128
in microorganisms, 129

Mutants, aminopurine induced, 104
double, in *Bacillus subtilis*, 54
induced, by base analogues, 102
mechanisms for induction of, 120
visible, in *Drosophila funebris*, 69
Mutational events, independence of, 53
"Mutation index," 141, 203
Mutation of two alleles, in *Antirrhinum*, 57
Mutation rates, ABO system and, 17-46
age dependence, 45
caffeine, and spontaneous rate in man, 226
dependence on division rate, 46
in mussels, 46-51
in strains LT2 and LT7 of Salmonella, 58
in yeast, in meiosis, 17
mistakes in, 4
sex dependence of, 16
Mutation rates in microorganisms, 55
"Mutation rate per generation," 58
Mutations, age dependence of, in man, 16
alkylating agents, 79
back, 2
5-bromouracil induced, 90
by a mistake in incorporation, 89, 94
caffeine-induced, 60
definition of, 1, 72
expression in man and in bacteria, 70
forward, 2
forward in yeast, 67
individual units of, 152, 156
induced, in phage, 83-85
induced by 2-aminopurine, 117
lethal, 70
operational terms for, 64
specificity in forward, 66
spontaneous, 136
"spontaneous" in man, 176
spontaneous, in Neurospora, 88
UV induced in *E. coli*, 132

Neighbor interaction, 154-55
Neurofibromatosis, 14
Neurospora, adenine plus inositol requiring strain of, 131, 134
lethal mutations in, 60
mutations, spontaneous in, 88
Nicotine, 169, 174, 195-96
Nitrate, 116
Nitrites, 80, 173
Nitrogen mustard, 169
Nitrous acid, 82-83, 88, 110, 149-51, 172, 179
deamination of nucleic bases by, 177
induction of mutation in bacteria by, 86
treatment of bacteria with, 85
Norethynodrel, 199-200
Nucleoside triphosphate, 104

Organic peroxides, 113, 124

Paraldehyde, 169, 172
Penetrance, 42
of gene, 34
Penicillium, mutagenic effect of manganese chloride in, 117
Pentobarbital, 202
Pericarp, growth patterns of, 51
Peroxidase, in microorganisms, 123
Peroxides, amino acid, 114
Peyotl, 176
pH, 146
Phage, action of ethylethane sulfonate on 111
induced mutations in, 149-53
in vitro, in vivo, 149
mutations, induced in, 83-85
Tessman's, 82
Phage T4, 82-83, 136
deletions in, 146-49
glucosylation, within the *rII* region, 143
incorporation of 2-aminopurine in, 91
quiet regions in, 139
revertants in, 144-46
spontaneous mutations, 138
Phaseolus, 33
Phenols, 172, 201
Phenoxybenzamine, 169, 171
Phenyl mercuric compounds, 199
Polycythemia, 35
Proflavine, 149
Progesterone, 199-200
Proline, 121

Purine riboside, antimutagenic effect of, 98
Pyridine, 201
Pyronine, 80

Quinacrine, 169, 174
Quinolines, 199

Recombination frequency, 139
Red cell, ghost, 23
Reduction division, in mice, 230
Reticulocytes, 36
Retinoblastoma, 14
Reversions, 152-55, 157
  adenine, 133
  histidine locus, *E. coli*, 134
  inositol, 133
  leucine, 133
  lysine locus, *E. coli*, 135
  tryptophan, 133
  tryptophan, in *E. coli*, 133
RNA, incorporation of P^{32} into, 33
  viral, deamination of cytosine, 59

Saccharomyces pombe, 67, 130
Salmonella, cysC region in, 118
  mutation rates in strains LT2 and LT7 of, 58
Scopolamine, 79
Sectorials, in maize, 51
Serine, 91
Sex ratio, age dependence of, 229
Sodium nitrite, 172
Somatic crossing over, in Drosophila, 56
"Specific locus phenotypes," 128
Spermatogonium, maturing of, 11
Steroids, 174
Streptomycin dependence, 117
Suppressor mutations, 142
Suppressors, intragenic, 160
Syndrome, Klinefelter's, 232
  Klinefelter's maternal age dependence of, 232
  Turner's, 232
  Turner's, maternal age dependence of, 232

TEM (tri-ethylene melamine), 105, 131
Temperature, 146
Teratogenesis, 170
Teratogens, 170
Tetramethyl uric acid, 188
Theobromine, 201
  metabolism of, 207
Theophylline, 201
Threonine, 58
Thymine, 95, 155
Thymonucleic acid, 81
Trace metals, 198
Transitions, 158
Translocations, 175
Transversions, 158
Triethylene imine, 80
Trimethyl uric acid, 188
Tryptophan, 54, 130
  reversions, in *E. coli*, 133
Tryptophan markers, *Bacillus subtilis* in, 55
Tryptophan reversions, 133
Turner's syndrome, 232
  maternal age dependence of, 232
Tyrosine, 172

Ultraviolet light, 67, 131, 133-34, 149
  induced mutations in *E. coli*, 132
Umbo, 46
Unstable genes, in *Delphinium ajacis*, 51
  in *Drosophila virilis*, 51-52

Valeric acid, 201
Variegation, in maize, 49
Virus, hepatitis, 174
Vitamins, 198
  B, 198

XO mice, induced by irradiation, 230
X-rays, 131

Yeast, forward mutations in, 67
  methionine cistron in, 53
  mutation rate in meiosis in, 17
  mutation rate of site met_{1-1} in, 56